(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 October 2002 (10.10.2002)

PCT

(10) International Publication Number
WO 02/079467 A2

(51) International Patent Classification[7]: C12N 15/10, 15/11, 5/10, C12Q 1/18, C07H 21/00, C12N 15/65

(21) International Application Number: PCT/DK02/00208

(22) International Filing Date: 26 March 2002 (26.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2001 00523 29 March 2001 (29.03.2001) DK

(71) Applicant *(for all designated States except US)*: **KØBENHAVNS UNIVERSITET** [DK/DK]; Nørregade 10, P.O. Box 2177, DK-1017 Copenhagen K (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **NIELSEN, Peter, E.** [DK/DK]; Hjortevænget 509, DK-2980 Kokkedal (DK). **GOOD, Liam** [CA/SE]; Sveavägen 164, B32, S-113 46 Stockholm (SE).

(74) Agent: **PLOUGMANN & VINGTOFT A/S**; Sankt Annæ Plads 11, P.O. Box 3007, DK-1021 Copenhagen K (DK).

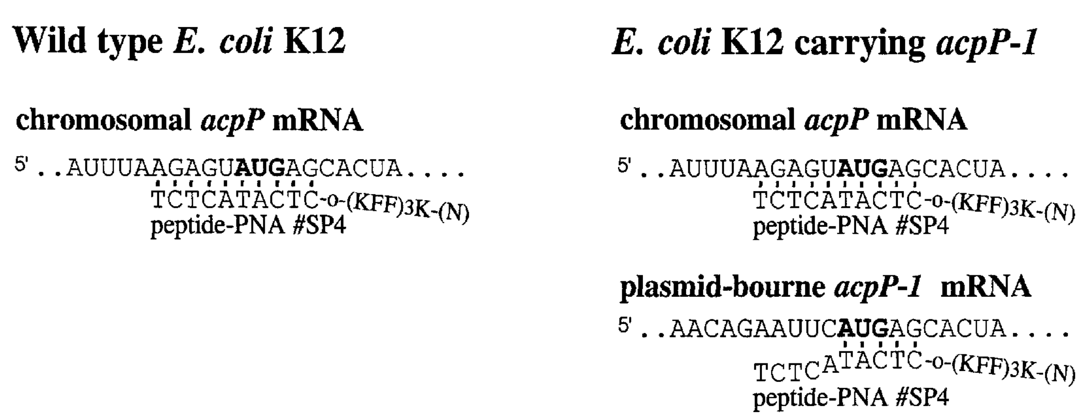
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EC, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTIBIOTIC-FREE BACTERIAL STRAIN SELECTION WITH ANTISENSE MOLECULES



(57) Abstract: A new method for an antibiotic-free selection of genetically modified cells is described. It is shown that antisense molecules targeted to an essential cellular gene inhibits growth and is suited as an agent for growth selection of cells transformed with a plasmid carrying an altered version of the essential gene. The results show that antisense molecules may be used for antibiotic-free selection of desired transformed microbes when targeted against an essential microbial gene. This technology is useful in genetic engineering for research growth and isolation of transformed organisms, and for industrial growth maintenance of transformed organisms, e.g. in the production of genetically engineered proteins as an environmentally safer alternative to traditional selection methods based on antibiotics.

WO 02/079467 A2

Antibiotic-free bacterial strain selection with antisense molecules

Field of invention

The present invention relates to a new method for an antibiotic-free selection of genetically modified cells. The method is based on the combination of antisense molecules, e.g. peptide nucleic acid (PNA) reagents, that are targeted against a growth essential cellular gene and the transformation of the cells with a recombinant DNA- cloning vector coding for a functional, but mutated version of said growth essential gene that is refractive to the antisense molecule.

General background

A basic and essential requirement for effective genetic engineering of bacteria and other cells propagated in cell cultures is the capacity to select for a cell with a specific genotypic alteration. The most common selection strategy in recombinant DNA technology is to include a cloned gene or DNA sequence in a genetic element (plasmid, virus, transposon etc.) that has a phenotypical property which allows for the separation of host cells containing the element (transformed cells) from cells that do not. Particularly useful is a gene that provides for survival selection. Thus, a selection of cells containing the wanted new genetic element can conveniently be achieved by growing cells on a medium containing a toxic substance and on which only the transformants expressing the "resistance gene" are able to survive.

While genes are available that provide for virus resistance, heavy metal resistance or polypeptide resistance, most current protocols for the selection of transformed cells are based on genetic elements (e.g. cloning vectors) that express genes coding for antibiotic resistance in the transformed host cell (see e.g. US 4,237,224; Ausubel, 2000). Illustrative antibiotics include penicillin tetracycline, streptomycin and sulfa drugs.

Although this strategy has proven to be very successful, a serious drawback with antibiotic resistant markers and the use of antibiotics in strain development and maintenance remains, namely the spread of antibiotic resistant pathogens, which in recent years has led to calls for reduced use of antibiotics in medicine, agriculture and industrial production (Davies, 1994). Furthermore, the emergence of multiple resistant organisms has seriously aggravated the situation by rendering even combination antimicrobial therapy inefficient (Walsh, 2000). It is therefore of central importance to develop alternatives to reduce the use of antibiotics in all areas.

Antisense RNA regulation is a natural mechanism for gene regulation, occurring in a wide range of organisms wherein an antisense RNA binds and inactivates a target mRNA molecule (Wagner & Simons, 1994). Much effort has been invested in developing synthetic antisense oligonucleotides that have eukaryotic cells as target, for both research applications and clinical uses (Wahlestedt, C. (1994) Trends Pharmacol Sci, 5, 42-6). Although it has been documented that antisense oligonucleotides can be effective in bacterial cells (WO 99/13893, Good, L. & Nielsen, P. E. (1998), Nat Biotechnol 16, 355-8, Good L. et al. (2000) Microbiology 146, 2665-70), surprisingly little effort has been invested in developing antisense oligonucleotides for microbes and no reports of using antisense technology in strain selection have so far emerged.

Peptide nucleic acid (PNA) is an antisense compound that has shown good results for specific gene targeting in a number of different organisms (Good & Nielsen, 1997; Larsen et al., 1999; Nielsen et al., 1991, WO 93/12129, US 5,773,571). In PNA compounds, the sugar backbone of an oligonucleotide is replaced with an amide containing backbone, in particular with an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al. (1991). In addition, the use of PNA oligomers as anti-sense oligomers for the treatment of diseases is taught by WO 93/12129 and U.S. 5,773,571; both are incorporated herein by reference.

In *E. coli,* PNA has successfully been targeted against both reporter genes and an essential gene (Good & Nielsen, 1998a; Good & Nielsen, 1998b). Furthermore, PNA molecules have shown good stability and high binding affinity, probably due to their uncharged pseudo-peptide backbone (Figure 1). As no general toxicity has been detected so far, PNA constitutes a good candidate for use as an antimicrobial drug, as taught by WO 99/13893. A major difficulty, though, has been the inadequate rate of uptake of PNA into bacterial cells. However, the uncharged and hydrophilic properties of PNA, as well as the peptide backbone have been helpful in improving cellular uptake. For example, it has been possible to link specific carrier peptides to the PNA backbone so that membrane passage in eucaryotic cells was facilitated (Pooga et al. (1998) Nat Biotechnol 16, 857-61).

Summary of the invention

The present invention addresses the above stated complications by providing a substantially new and inventive method for selecting for a transformed cell without using antibiotics.

The invention is based on the observation that it is possible to design antisense molecules that specifically inhibit the expression of an essential cellular gene, but which do not inhibit the expression of a mutated, but functional homologous version of the same gene. When the mutated, but functional version of the essential gene is incorporated into a cloning vector and the vector is introduced into cells, cells containing the vector will survive treatment with the antisense molecule, because the mutated, but functional homologous version of the essential gene will provide the essential function to the cells, whereas cells that do not contain the vector will die. Thus it is possible to use antisense molecules to select for transformed or transfected cells without the common use of antibiotics.

A wide variety of types of antisense molecules and suitable essential genes of both prokaryotes and eukaryotes are described.

In a preferred embodiment of the invention, the antisense molecules are short antisense molecules that are able to down modulate bacterial gene expression both *in vitro* and/or *in vivo*. In another equally preferred embodiment of the invention, the short antisense molecules are additionally complexed with cell wall or cell membrane transport peptides, thus improving the uptake of the antisense molecule into the cell.

In an particular preferred embodiment, antisense peptide nucleic acids (PNA) are covalently conjugated with cell wall or cell membrane transport peptides. Such peptide-PNA conjugates directed against various essential genes of *Escherichia coli (E. coli)* are herein shown to be able to inhibit bacterial growth both of permeable *E. coli* strains, such as the AS19 strain, and of *E. coli* K-12 strains that are wildtype with respect to permeability, as well as of *Bacillus subtillis*.

It is demonstrated that an antisense molecule directed against the essential *acpP* gene encoding the acetyl carrier protein Acp, which is central for fatty acid biosynthesis in *E. coli* (Cronan, 1996), can be used as a reagent with good antibacterial effects.

In a further preferred embodiment, antisense PNA targeted against the essential gene *acpP* is used to select transformed *E. coli* cells which contain a plasmid carrying an

altered *acpP* gene, in a mixed population consisting of both transformed and non-transformed cells. The surprising results found by the present inventors led to the conclusion that antisense PNA can be used for antibiotic-free antimicrobial selection when said antisense PNA is targeted against an essential cellular gene.

The use of *acpP* mRNA-targeted PNA as a selective agent for an *E. coli* cell that is transformed with a genetic element carrying a mutated, but functionally homologous version of said gene, was furthermore demonstrated by selection of plasmid carrying cells after electrotransformation. The results show that antisense PNA can substitute the use of antibiotics and other environmentally problematic substances as selective agents in gene cloning procedures.

The invention provides several avantages to microbial selection systems, as well as to other non-microbial selection systems, as described in the prior art. The possibility to select and maintain a plasmid containing bacterial culture without the use of antibiotics can be used for a wide variety of applications within the fields of research, development and industrial production, involving genetically modified micro-organisms. Another important aspect of the present invention is the use of embodiments of the present invention for inhibiting bacterial infections in eukaryotic cell cultures. Yet another important aspect of the present invention is to use antisense constructs to "purify" or "decontaminate" microbial cultures used in industrial applications. In the diary and brewing industry it is of paramount importance to initiate and retain fermentation free of contaminating microorganisms.

Detailed disclosure of the invention

The present intention relates to a novel method for antibiotic-free selection of transformed cells that is based on antisense molecules targeted against essential cellular genes.

"Antibiotic-free selection" in the present application refers to a situation wherein an efficient selection is obtained without use of antibiotics, such as tetracycline or ampicillin.

The present invention thus provides a method for selecting "genetically modified cells", wherein the selection is performed by modifying cells containing a growth essential gene X, inserting in such cells a "construct" containing a gene Y that is a mutated, but functionally homologous version of gene X (X'), and treating said modified cells with an antisense or antigene construct directed against said essential gene X of the cells, thereby obtaining a preferential growth of the modified cells over other, non-modified cells.

In the present context "genetically modified cells" are defined as cells that are derived from "wild-type" cells ("wild-type" cells being reference, or standard cells) that are mutated by introducing a "genetic element". The term "genetic element" generally means a polynucleotide comprising a region that encodes a polypeptide, part of a polypeptide, or a region that regulates transcription or translation or other processes important to the expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression.

Genetic elements comprise a polynucleotide molecule that replicates as an episomal element; that is, as a molecule physically independent of the host cell genome. They may be described as plasmid, virus, retrovirus, bacteriophage, cosmid, artificial chromosome (bacterial or yeast), or a nucleic acid sequence that behaves as an autonomous unit of polynucleotide replication within a cell. One subset of genetic elements are the so-called "cloning vectors".

A "cloning vector" or "vector" is herein defined as a plasmid, virus, retrovirus, bacteriophage, cosmid, artificial chromosome (bacterial or yeast), or nucleic acid sequence which is able to replicate in a host cell, characterized by one or a small number of restriction endonuclease recognition sites at which the sequence may be cut in a predetermined fashion, and which may contain an optional marker suitable for use in the identification of the cells that have taken up the vector, i.e. the "transformed" cells, e.g., tetracycline resistance or ampicillin resistance. Included in the definition of "cloning vector" or "vector" is the specific type of nucleic acid sequence that is not able to replicate in the

target or final host cells but which – under selective pressure – can be inserted into chromosomes and are thereafter maintained without selective pressure. Often such non-autonomously replicating or nucleic acid sequences contain or are part of vectors that contain sequences allowing them to replicate in a suitable secondary host cell. A cloning vector may or may not possess the features necessary for it to operate as an "expression vector" i.e. a vector that facilitates the transcription and/or translation of an appropriately inserted gene. In the present context the term "construct" refers to a specific cloning vector with one or more specific polynucleotide fragments inserted in an ordered fashion.

A "growth essential gene" is herein defined as a gene whose inactivation is lethal to the organism, or alternatively, whose inactivation will inhibit or disturb the natural proliferation rate of an organism.

"Antisense molecules" or "antisense reagents" can, in the present context, be any molecule that hybridizes by a sequence specific base pairing to a complementary DNA and/or RNA sequence. In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays, and in the case of *in vitro* assays, under conditions in which the assays are performed. Typical antisense molecules" or "antisense reagents" are any oligonucleotide, such as DNA, RNA, any peptide nucleic acid, any other nucleic acid derivative, or mimic and/or derivative thereof. The target sequence is not restricted to the "sense" or "coding" strand of mRNA, although this is often the target. The present invention employs "antisense compounds", or "antisense constructs" which are used interchangeably in the present text. In particular, the use of PNA and oligonucleotides, for use in modulating the function of nucleic acid molecules encoding essential genes is addressed. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding said essential gene. As used herein, the terms "target nucleic acid" and "nucleic acid encoding said essential gene" encompass a DNA encoding said gene, and/or an RNA (including pre-mRNA and mRNA) transcribed from such DNA. The

specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense" (when the target is RNA) or "antigene" (when the target is DNA). The functions of DNA to be interfered with include replication and transcription. This effect is referred to as "antigene". The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA and is referred to as "antisense". However, the distinction between "antisense" and "antigene" is not absolute. The overall effect of such interferences with target nucleic acid function is a specific modulation of the expression of said essential gene. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression.

Gene Y on the vector is ideally able to complement the essential cellular gene X. In the present context, a "complementing" gene is a gene that by its presence is able to restore the phenotype of a mutated cell. To restore the phenotype of a mutated cell, the complementing gene does not need to be identical to an unmutated version of said gene. There are several examples in the art describing mutated versions of a gene that is able to complement a mutated gene. One particular example is the so-called alpha-complementation of the *E. coli lacZ* gene. Usually, the mutated gene and the mutated complementing gene are mutated in different positions, the result being a restoration of the function of the non-mutated gene through complementation. In the present invention, the essential cellular wild-type gene is not mutated but inactivated through the specific interaction of the antisense construct. The viable phenotype is restored by the presense of an altered but yet functionally equivalent version of said essential gene. Thus, the altered version of the essential gene is sufficiently different in nucleotide sequence to be refractive to the inhibitory effect of the antisense construct.

In the situations where gene Y on the vector is a mutated but functionally equivalent of the essential cellular gene X, it is referred to as X'. The mutated gene X' on the vector can e.g. be a "silent mutation" of gene X. In the present context, a "silent mutation" is a mutation which does not lead to a change in the product encoded by the gene.

The present invention thus discloses a method, wherein said antisense or antigene construct down-regulates the expression of the growth essential gene X, thereby exercising a detrimental effect on the untransformed cells in the mixed culture.

In the present invention, antisense molecules can be selected from the group consisting of oligonucleotides, oligonucleotide analogues, oligonucleotide mimics, such as for example PNA, locked nucleic acids (LNA), phosphorothioate, 2'-methoxy-, 2'-methoxyethoxy-, morpholino, phosphoramidate oligonucleotides or the like. In the present invention, antigene molecules can furthermore be selected from the group consisting of triplex forming or strand invading oligonucleotides, oligonucleotide analogues, oligonucleotide mimics, such as for example PNA, locked nucleic acids (LNA), phosphorothioate, 2'-methoxy-, 2'-methoxyetyhoxy-, morpholino, phosphoramidate oligonucleotides or DNA minor groove binding polyamides (oligo pyrroles/imidazoles etc.) as described (Gottesfeld JM et al. 2000; Dervan PB et al. 1999) or the like.

The term "oligonucleotide(s)" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages which function similarly or combinations thereof. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases and other enzymes, and are in the present context described by the terms "oligonucleotide analogues" or "oligonucleotide mimics".

The present invention also comprises other oligomeric antisense compounds, including, but not limited to, oligonucleotide mimetics such as described below.

The antisense compounds in accordance with this invention preferably comprise from 4 to 30 nucleobase units. The term "nucleobase units" is used in the present text to describe both the number of nucleotides in a oligonucleotide and the number of nucleobase-carrying monomers of a oligonucleotide mimetic. Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from 7 to 15 nucleobases. As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric

structure can be further joined to form a circular structure; however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorous atom in the backbone and those that do not have a phosphorous atom in the backbone. For the purposes of this specification, modified oligonucleotides that do not have a phosphorous atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogues of these, and those having inverted polarity, wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorous-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorous atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino

backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

Other embodiments of the invention consist of oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2 --NH--O--CH_2 --, --CH_2 --N(CH_3)--O--CH_2 -- [known as a methylene (methylimino) or MMI backbone], --CH_2 --O--N(CH_3)--CH_2 --, --CH_2 --N(CH_3)--N(CH_3)--CH_2 -- and --O--N(CH_3)--CH_2 --CH_2 -- [wherein the native phosphodiester backbone is represented as --O--P--O--CH_2 --] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also comprised are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Other modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotides. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-

halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937).

Representative U.S. patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

Other embodiments of the invention comprise oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2 --NH--O--CH_2 --, --CH_2 --N(CH_3)--O--CH_2 -- [known as a methylene (methylimino) or MMI backbone], --CH_2 --O--N(CH_3)--CH_2 --, --CH_2 --N(CH_3)--N(CH_3)--CH_2 -- and --O--N(CH_3)--CH_2 --CH_2 -- [wherein the native phosphodiester backbone is represented as --O--P--O--CH_2 --] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also comprised are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O--, S--, or N-alkyl; O--, S--, or N-alkenyl; O--, S-- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_3 to C_3O alkyl or C_2 to C_3O alkenyl and alkynyl. Particularly preferred are O[(CH_2)$_n$ O]$_n$ CH_3, O(CH_2)$_n$ OCH_3, O(CH_2)$_n$ NH_2, O(CH_2)$_n$ CH_3, O(CH_2)$_n$ ONH_2, and O(CH_2)$_n$ ON[(CH_2)$_n$ CH_3)]$_2$ where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_3 to C_3O lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2 CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl group. A preferred modification includes 2'-methoxyethoxy (2'--O--CH_2 CH_2 OCH_3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S.

patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference.

Preferred embodiments of the invention are oligonucleotide mimetics, in which both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, and which is the most preferred embodiment of the present invention is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an oligonucleotide is replaced with an amide containing backbone, preferably through a methylene carbonyl linker, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

By "PNA" is meant any peptide or peptide conjugated molecule comprising one or more PNA monomers. Also molecules containing fully or partially modified PNA are considered PNA.

In another embodiment of the invention, the oligonucleotide mimetics are a bi-cyclic DNA analogue called Locked Nucleic Acid (LNA) (see International Patent Application WO 99/14226; P. Nielsen et al, J. Chem. Soc., Perkin Trans. 1, 1997, 3423; P. Nielsen et al., Chem. Commun., 1997, 9, 825; N. K. Christensen et al., J. Am. Chem. Soc., 1998, 120, 5458; A. A. Koshkin et al., J. Org. Chem., 1998, 63, 2778; A. A Koshkin et al. J. Am. Chem. Soc. 1998, 120, 13252-53; Kumar et al. Bioorg, & Med. Chem. Lett.,1998, 8, 2219-2222; and S. Obika et al., Bioorg. Med. Chem. Lett., 1999, 515). Interestingly, incorporation of LNA monomers containing a 2'-O,4'-C-methylene bridge into an oligonucleotide sequence led to an pronounced improvement in the hybridisation stability of the modified oligonucleotide (see above and e.g., S. K. Singh et al., Chem. Commun., 1998, 455).

"LNA" in the present context means any oligonucleotide either fully or partially modified with LNA monomers. By LNA monomers, any monomeric unit is meant that comprises the LNA bi-cyclic structure.

A preferred embodiment of the present invention thus relates to a method for selecting genetically modified cells, wherein the selection is performed by modifying cells containing a growth essential gene X with a construct containing a gene Y, and treating said modified cells with an antisense or antigene construct directed against said essential gene X of the cells, thereby obtaining a preferential growth of the modified cells over other, non-modified cells,

A particularly preferred embodiment of the present invention thus relates to a method for selecting genetically modified cells, wherein the selection is performed by modifying cells containing a growth essential gene X with a construct containing a gene Y, and treating said modified cells with an antisense or antigene construct directed against said essential gene X of the cells, thereby obtaining a preferential growth of the modified cells over other, non-modified cells, wherein said antisense construct is a PNA.

Said antisense construct can additionally be a modified PNA, wherein the modification is e.g. obtained by linking the PNA to a peptide, a carbohydrate or a lipid or wherein the modification is obtained by internal modification, i.e. backbone modification, in which the glycine of the PNA backbone is exchanged for other α-amino acids. In a particularly preferred embodiment of the present invention, the PNA is covalently linked to the KFFKFFKFFK peptide. As seen in Table 5, the presence of the KFFKFFKFFK (SEQ ID NO: 1) peptide substantially increases the efficacy of the PNA-antisense construct.

In general smaller molecules can permeabilize cells more efficiently than larger molecules. Antisense PNAs are larger than most drugs, e.g. antiobiotics. Thus PNA size or length is likely to be an important parameter for the permeabilizing and thus the efficacy. To evaluate the size/activity relationship for antisense PNAs in *E. coli* cultures, PNAs targeted to the start codon region of the chromosomal β-galactosidase gene (*lacZ*) (see Figure 9 and Table 5) were synthesized over the 7 to 15 mer size range. Inhibition of *lacZ* expression was determined using an *E. coli* liquid culture assay (for details see Good and Nielsen 1998a and 1998b, Good et al. 2001). The results are illustrated for a wild type *E. coli* strain (K12) and a permeable mutant (AS19) (Figure 10). In both strains, PNAs in the 9 - 12 mer range were most active. The 7 and 8 mer PNAs tested were the least active. Also, truncated versions of the triplex-forming PNA directed against ribosomal RNA were tested, and the shorter versions were not found to increase potency significantly in this case (Table 5: PNAs 1406, 1407 & 1410 versus PNA 1143). These results are consistent

with reports of impressive antisense effects with even shorter oligomers (Wagner et al., 1996). Binding affinity to the RNA target increases with length, whereas uptake efficiency is expected to decrease dramatically with length. Also, shorter oligomers naturally offer higher sequence specificity at the expense of gene target specificity. Finally, as an initial test of selectivity, a mismatched 12 mer PNA (PNA 1878 versus PNA 1834) was tested and found to be significantly less active than the fully matched version (Table 5; Figure 10).

Thus, an especially preferred embodiment of the present invention method relates to a method for selecting genetically modified cells, wherein the selection is performed by modifying cells containing a growth essential gene X with a construct containing a gene Y, and treating said modified cells with an antisense or antigene construct directed against said essential gene X of the cells, thereby obtaining a preferential growth of the modified cells over other, non-modified cells, and wherein said antisense construct contains a PNA consisting of 4-30 nucleobase units, such as 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 nucleobase units. Most preferred embodiments comprising an antisense construct that contains a PNA consisting of 10-12 nucleobases, but equally comprised in the scope of the present invention, are embodiments wherein said antisense construct contains a PNA consisting of 7-15 nucleobase units.

It is an inherent feature of most PNA molecules that they are very stable in most biological settings. In addition, PNA has proved to possess very low general toxicity. To evaluate the toxicity of PNA to eucaryotic cells, eucaryotic HeLa cell cultures were infected with 10^4 colony forming units (cfu) of wild type *E. coli* K 12 and antisense peptide-PNA conjugate directed against the acpP of *E. coli* was added. Whereas PNA at a concentration of 2 μM or higher fully cured the HeLa culture of *E. coli* infection, no visible effect was seen on the HeLa cells even at the highest concentration tested (20 μM).

The *E. coli* outer cell wall is a major barrier to PNA (Nikaido, 1994). However, bacteria are permeabilized by cationic antimicrobial peptides and such compounds can act synergistically with antimicrobials that enter cells poorly (Hancock, 1997). As shown herein, *E. coli* cells permeabilized by the polymyxin nonapeptide are more susceptible to PNAs. Covalent attachment of such a permeabilizing agent can further improve cell entry, as has been demonstrated in related applications with eukaryotic cells (Schwarze, 2000; Aldrian-Herrada, 1998; Pooga, 1998; Cutrona, 2000). Instead of the polymyxin nonapeptide (Good, 2000) a quite simple synthetic peptide (KFFKFFKFFK (SEQ ID NO: 1)) was recently shown by Vaara (1996) to facilitate uptake of antibiotics into bacteria without any inherent antimicrobial activity. To test whether this cell wall permeating peptide, when conjugated to PNA oligomers, could enhance the uptake and thus efficacy of antisense

PNAs, 12 and 15 mer anti-*lacZ* PNAs were synthesized with a KFFKFFKFFK (SEQ ID NO: 1) peptide attached via a flexible ethylene glycol linker (Table 5). Both 12 and 15 mer conjugates were 15-20 fold more potent than the corresponding PNAs without attached peptides (Table 5; Figure 11). Also, consistent with the length comparison experiment, the 12-mer peptide-PNA conjugate was significantly more active than the 15-mer version. Furthermore, the free peptide did not inhibit LacZ production (e.g. via general toxicity), nor did a mixture of free peptide and naked PNA. Thus covalent attachment of PNA to the carrier peptide was needed for efficient antisense effects (Table 5; Figure 11).

To evaluate the specificity of the more potent anti-*lacZ* peptide-PNAs, one 15 mer (SP183 versus 1873) and two 12-mer mismatched peptide-PNA conjugates (1901 & SP182 versus 1900) were included in the study. These control constructs showed poor *lacZ* inhibition when compared to the fully matched constructs of similar size and composition. Also, the anti-*lacZ* constructs showed very low inhibition of a second reporter gene (luciferase) and low general toxicity (Table 5). Therefore, the peptide-PNAs were seen to retain good overall specificity.

Furthermore, *lacZ* antisense constructs can be an antisense molecule, such as a PNA, that is covalently linked to a cell wall or cell membrane transport peptide to improve its uptake into a cell. Examples of such cell wall or cell membrane transport peptide are peptides comprising the amino acid sequence: KFFKFFKFFK (SEQ ID NO: 1) or KFFKFFKFFKC (SEQ ID NO: 2) or IKFLKFLKFLC (SEQ ID NO: 3) or VDKGSYLPRPTPPRPIYNC (SEQ ID NO: 4) or ILPWKWPWWPWRRGC (SEQ ID NO: 5) or KLAKALKKLLC (SEQ ID NO: 6). In a preferred embodiment of the invention, the antisense construct is a PNA covalently linked to a cell wall or cell membrane transport peptide comprising the amino acid sequence: KFFKFFKFFK (SEQ ID NO: 1).

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. The antisense compounds

used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The present invention also provides a method for selecting for transformed cells that employs antisense molecules directed to the site of the start codon of an essential prokaryotic or eukaryotic gene X, and wherein the result of the interaction of said antisense with the mRNA of said essential gene is an inhibition of the translation of said essential gene X. The term "start codon" or "initiation codon" are herein used interchangeably. The start codon is the first mRNA triplet to be translated during protein or peptide synthesis and immediately precedes the structural gene being translated. The start codon is usually AUG, but may sometimes also be GUG or UUG.

An essential gene of the present invention is typically selected from, but not limited to, the group of genes consisting of essential genes of *Escherichia* species and subspecies including *E. coli* and *E. coli* K12, *Bacillus* species and subspecies including *B. subtilis, Saccharomyces* species including *S. cerevisiae* and *S. carlsbergensis, Schizosaccharomyces* species including *Schizosaccharomyces pombe, Lactococcus* species including *Lactococcus lactis* and *Lactococcus lactis* subsp. *cremoris*, *Streptococcus* species, *Enterococcus* species, *Lactobacillus* species, *Leuconostoc* species, *Oenococcus* species, *Pediococcus* species, *Bifidobacterium* species, *Pseudomonas* species including *Pseudomonas aeruginosa* and *Pseudomonas acidovorans*, *Chromobacterium* species such as *Chromobacterium violaceans*, *Kluyveromyces* species including *K. lactis*, *Pichia* species including *Pichia pastoris*, *Hansenula* species including *Hansenula polymorpha*, *Yarowia, Schwaniomyces* and *Zygosaccharomyces* species. Most of these organisms are of major industrial value as persons skilled in the art will know. Many of the organisms find important use in the industrial production or processing of food. Within this field of industrial activity, it is of paramount importance that the level of antibiotics used during the activity is extremely low or nil. The present invention offers an efficient solution to the problem of producing food using genetically modified organisms, while leaving very little or no trace of antibiotics in the product.

An essential gene X is therefore preferably selected from, but not limited to, the group of genes consisting of *E. coli* genes: *acpP, accD, acpS, frr, infA, lnt, murA, murI, parC, proS, rpoE* and *rpsB, and/or Bacillus subtilis* genes *acpA, sigH, nadE, sbp, ftsZ, sipS, sigA, tagH*

and *tagG and/or Saccharomyces cerevisiae* genes ACC1,TOP2, ALA1, CDC4, CDC28, RPC25, PMI40, PAP1, TFC8, YKT6, TAF145 and TIF11.

In yet a specific embodiment of the present invention, a method for selecting genetically modified *E. coli* cells is provided, wherein said modified cells are treated with an antisense or antigene construct directed against the essential *acpP* gene of E.coli, and wherein said antisense or antigene construct reduces the level of expression of the growth essential *acpP* gene, thereby excercising a bacterioicidal and/or bacteriostatic effect on the untransformed cells in the mixed culture. In said embodiment of the present invention, the antisense construct can for instance comprise the nucleobase sequence: (5'-/N-)CTCATACTCT(3'-/C-) as shown in SEQ ID NO: 7, or (5'-/N-)CATACTCTTAAA(3'-/C-) shown in SEQ ID NO: 8, or (5'-/N-)CCTATCAAAACT(3'-/C-) as seen in SEQ ID NO: 9.

Gene Y on the vector is ideally able to complement the cellular gene X because gene Y is a mutated, but functionally equivalent version of gene X, herein defined as X'. In the present context, gene X' typically stands for a gene coding for the wild-type product of gene X, such as for the *acpP* gene, which is mutated at one or more positions between the Shine-Delgarno sequence (ribosome binding site) and the start codon of the gene. One example of a gene X' comprises the sequence 5'-AACAGAATTCATGAGCACTA-3' (SEQ ID NO: 19).

The present inventors have previously shown that a triplex-forming PNA oligomer (PNA 1143) targeted to the α-sarcin loop of the 23S ribosomal RNA inhibits the growth of *E. coli*. (Good, 1998). The attached peptide was shown also to improve the potency of such anti-microbial PNAs, when a KFFKFFKFFK (SEQ ID NO: 1) peptide was conjugated to this PNA. The "naked" version of this anti-ribosomal PNA inhibited translation but only affected cell growth of a permeable *E. coli* strain (AS19) in diluted growth media. In contrast, the new anti-ribosomal peptide-PNA conjugate (PNA1934) exhibited an MIC of 3 μM against *E. coli* K12 grown in Mueller Hinton broth and an MIC of 700 nM when grown in dilute broth (Table 5). Again, free peptide and PNA 1143 were not inhibitory at these concentrations, showing that conjugation of the peptide with PNA is needed for potent growth inhibitory effects.

The results presented (e.g. Table 5) clearly show that the linker connecting the peptide and the PNA is not "a silent player". In particular the flexible ethylene glycol-lysine linker (eg1) seems advantageous illustrated by the fact that the PNA construct assembled via maleimide SMCC coupling (PNA 1978) is more than 10 fold less potent than the ethylene glycol linked PNA (PNA 1900). Also, peptides other than the KFFKFFKFFK (SEQ ID NO: 1) motif are able to carry PNA oligomers into bacteria as indicated by the comparable potencies for PNAs 1978, p29, p30, p31 and p32. Thus it should be possible to both

optimize the linker strategy and discover improved peptide carriers to further improve antisense effects. However, at present good antibacterial effects have been obtained with both the H-KFFKFFKFFK-eg1-jtjtjjt-(eg1)$_3$-tcctctc- lysNH$_2$ (SEQ ID NO: 21; PNA 1934) and the H-KFFKFFKFFK-eg1-ctcatactct-NH$_2$ (SEQ ID NO: 20; PNA SP4) construct, wherein "j" indicate pseudo-isocytosine bases and "eg1" is the ethylene glycol linker called "AEEA-OH spacer" and identified by Cat# GEN063030 of AppliedBiosystems, Framingham, MA, USA.

In the present invention, the potential for PNAs targeted against mRNA was shown as exemplified by a PNA complementary to the start codon region of the *acpP* gene. This anti-*acpP* peptide-PNA conjugate (SP4) showed an MIC against *E. coli* K12 of 1 μM in rich broth and an even lower MIC in dilute broth (Figure 12A, Table 5). Antisense specificity of the anti-*acpP* peptide-PNA was demonstrated in two ways. First, a control peptide-PNA with a scrambled PNA sequence (SP181, SEQ ID NO: 51) showed 15 fold higher MIC than the fully matched version SP4 (Table 5). This was consistent with an *acpP* specific inhibitory effect for SP4. Second, to confirm that growth inhibition was due to down regulation of *acpP* expression, the *E. coli acpP* gene was cloned and selected bases were altered to eliminate the potential for PNA binding, but still encoding native *acpP*. Transformation of *E. coli* cells with this target-site-altered version of *acpP* rescued the bacteria from PNA growth inhibition (Figure 12B). Complementation by the altered *acpP* allele gives strong genetic evidence for specificity and confirms an antisense mechanism of action for the anti-*acpP* peptide-PNA. As a tool for genetics, the experimental approach can be extended to a wide variety of experimental questions.

The effects of antibacterial peptide-PNA conjugates on *E. coli* cells were further examined by assessing cell viability. Within one hour of exposure to 2 μM anti-*acpP* PNA SP4, the number of colony forming units decreased dramatically from 10^5/ml and no viable cells were detected after three hours, showing that the PNA treatment was bactericidal (Figure 13). Again, the strain containing the binding-site-altered allele was resistant to PNA. These results are encouraging for antimicrobial development as bactericidal activity is desirable and such effects on viability by antisense peptide-PNAs can be used to evaluate microbial genes for suitability as drug targets.

To evaluate any possible anti-*acpP* PNA (SP4) toxicity to eukaryotic cells, as well as to examine the anti-bacterial potential of antisense inhibition in the presence of eukaryotic cells, an anti-*acpP* PNA construct was tested against *E. coli* grown in cell culture medium. In this medium, which should be relevant for any *in vivo* application, the PNA is 10 fold more potent (MIC = 200 nM) than in MH broth (Table 5). Further, HeLa cell cultures were infected with 10^4 cfu of non-invasive *E. coli* K12 and PNA was added immediately post infection. This system can be viewed as a model for the growth of an extracellular

pathogen in a host. The PNA did not visibly affect HeLa cell growth at the highest concentration tested (20 μM) (Figure 14), and PNA at 2 μM or higher appeared to fully cure the HeLa culture of the infection. No live bacteria were detected from these samples in an agar colony forming unit test, confirming that the anti-*acpP* PNA is bactericidal under these conditions. Again, the sequence specificity of the effect was evaluated in cultures infected with *E.coli* carrying the binding-site-altered *acpP* allele (K12-*acpP-1*) and this infection was resistant to PNA up to at least 20 μM (Figure 14). Antisense PNAs are larger than most antimicrobials and the conjugation of a peptide carrier further increases the molecular weight to approximately 4000. Therefore, a 2 μM treatment corresponds approximately to 8 μg/ml, which is somewhat high compared to conventional antibiotics. Nevertheless, the observed antimicrobial effects within a cell culture system broaden the potential scope for the approach and raise the prospects for PNA-based antibacterial drugs.

As shown in the present invention, nanomolar concentrations of PNA directed towards the essential *E. coli acpP* gene not only inhibited bacterial growth, but also had a strong bactericidal effect. This shows that it is possible to replace antibiotic use with PNA. Similarly, it is demonstrated that PNA directed towards the *Bacillus subtilis acpP* homologue infer complete growth arrest and strong bactericidal effects (example 10). This results open possibilities to use antisense PNAs for strain selection and construction in research and for industrial applications in a wide range of bacteria.

To test the specific growth inhibitory properties of *acpP* targeted PNA, wild type *E. coli* K12 cells were grown together with a K12 strain carrying a plasmid with a target site altered copy of the *acpP* gene (K12/*acpP-1*). Figure 3 is a graphical representation of this experiment. Selection was carried out using a 1 μM concentration of anti-*acpP* PNA (PNA SP4). The results shown in Table 2 indicate a very good selective effect upon cells in a mixed culture. A strong selection effect is seen after just one hour (83.3% Amp^r clones as compared to 50% in the start culture) and 100% selection efficiency was obtained within only two hours. As shown before, a strong effect of PNA upon wild type K12 cells is seen when grown as a monoculture. The rescue effect previously reported is also seen for the K12/*acpP-1* strain grown with 1 μM PNA.

The strong selection efficiency in a mixed bacterial culture suggests that antisense PNA selection of transformants is also possible. Electroporation was chosen as the transformation method, and electrocompetent *E. coli* DH5α were used. About 10^8 bacteria were transformed with 30 ng pKK223-3/*acpP-1* DNA. After recovery in SOC media for 40 minutes and dilution in 10% LB, low salt selection was carried out with a 1 μM concentration of wild type *acpP*-targeted PNA. Within 6 hours (see Table 3) the selection efficiency was almost complete (96.6% Amp^r), and after 18 hours the culture contained

only ampicillin resistant bacteria. The genotypic identification revealed that all tested LBA-growing clones (11/11) contained a plasmid of identical size as pKK223-3 (see Figure 4). The results of this study show convincingly that antisense PNA can be used to select for specific strains from a mixed *E. coli* culture when targeted against an essential gene. Also, PNA can be used for antibiotic-free selection of transformed cells in spite of a relatively low amount of viable transformants compared to the large background amount of non-transformed bacteria. This opens a possibility to use PNA as an alternative for antibiotic resistance markers in genetic cloning procedures. A major advantage will thus be a reduced usage of antibiotics and thereby a decreased risk for the spread of antibiotic resistance traits to pathogens.

Legends to figures

Figure 1.

Chemical structure of DNA and PNA. In PNA the phosphodiester backbone is replaced with a pseudo-peptide backbone, which provides similar spacing between the bases (marked as b) as in DNA. Hybridisation of antisense PNA oligonucleotides results in a stable helical complex and follow Watson-Crick pairing rules.

Figure 2.

Anti-*acpP* PNA complementary to wild type and mutant *acpP* mRNA. The structure of the wild type chromosomal and mutant plasmid-borne copies of the *acpP* genes are shown, along with the antisense PNA, which is complementary only to the native *acpP* mRNA start codon. Cells harbouring the mutant *acpP* allele are not affected by the presence of PNA whereas growth is arrested for wild type *E. coli* (as shown in Good et al. in press).

Figure 3.

Flow chart of PNA selection for pKK223-3/*acpP-1* carrying bacteria. Pre-grown *E. coli* K12 and K12/*acpP-1* were diluted in 10% LB low salt media, and 10^4 cells of each dilution were added either separately or as a mixture to the wells of an "Ultra low attachment 96 well plate with lid". PNA was added to a final concentration of 1.0 μM and the cultures were adjusted to 100 μl with 10% LB low salt. The 96 well plate was incubated in 37°C with shaking every 5 minutes and 2 μl samples were harvested after 0, 1, 2, 3, 4, 6, 8, 16 and 24 hours. The harvested cells were diluted in water to different concentrations and 50 μl of each dilution was plated out on LB-plates. The LB-plates were incubated at 37°C for 48 hours and an estimation of the total number of cfu was performed by counting the colonies formed. Colonies derived from each harvest time were transferred one by one

to LBA-plates that were then incubated for 24 hours at 37°C. Only cells containing pKK223-3/*acpP-1* would be able to grow since the plasmid contains the ampicillin resistance gene. The number of growing clones were counted and related to the total number of transferred clones (Table 2). After electrotransformation of *E. coli* DH5α with pKK223-3/*acpP-1,* the cells were left to recover in SOC media at 37°C for 40 minutes. The bacteria were diluted in 10% LB low salt and 10^4 cells were added to the wells of an "Ultra low attachment 96 well plate with lid" along with PNA SP4 (1.0 μM final concentration) to a total volume of 100 μl. Again, the plate was incubated and cells were harvested, plated and replated as described above (Table 3).

Figure 4.

Genotypic identification of PNA selected strains. Plasmids were purified from colonies that grew on LBA-plates. The samples were loaded on a 1% agarose gel. Lanes 1 and 14 contain the marker λ *Hind* III, lanes 2-7 and 9-13 contain samples derived from LBA-growing colonies, and lane 8 is a control with plasmid pKK223-3/*acpP-1*.

Figure 5.

Map of plasmid pKK223-*acpP*-1. The insert shown at the top indicates the cloning strategy, construction and the sequence that replaces the native *acpP* start codon region (see also Figure 2). The arrows indicate antibiotic resistance markers, the Ptac promoter and the origin or replication from pBR322 is indicated.

Figure 6.

Scan of the *acpP* mRNA for target sites susceptible to inhibition by antisense peptide-PNAs. The 5' region of the *E. coli acpP* mRNA is shown, representing the X-axis. The binding site of the antisense PNAs is shown as lines below the mRNA sequence, along with the name corresponding to the peptide-PNA. The lines within the graph indicate the minimum inhibitory concentrations (MIC) determined for each peptide-PNA as indicated on the Y-axis. Peptide-PNAs found to not be inhibitory at the highest concentration tested are indicated at the top.

Figure 7.

The green fluorescence expression plasmid pSPT-Pxyl-GFP. The insert shown at the top indicates the cloning strategy, construction and peptide-PNA 1900 binding site. The arrows indicate antibiotic resistance markers, the xylose promoter and the origin or replication from pBR322 is indicated.

Figure 8.

Green fluorescence protein expression and antisense inhibition in B. subtilis 168. After overnight growth in the presence or absence of PNA, *B. subtilis* cells were visualised by light microscopy at 100 x magnification. The top panels illustrate control cells that did not carry the *gfp* reported gene. Cells expressing *gfp* were treated with PNA as indicated.

Figure 9.

Wild type chromosomal *E. coli* β-galactosidase (*lacZ*) mRNA start codon target region and examples of antisense PNAs. N: indicates the PNA amino terminus, corresponding to the 5' end of a conventional oligonucleotide. The start codon AUG is underlined.

Figure 10.

LacZ expression and inhibition in *E. coli* with a range of differently sized PNAs. The histograms at the left illustrate results for wild type strain K12 and those at the right are for the permeable mutant strain AS19. Values represent LacZ activities relative to control levels for cultures grown in 10% LB broth. With reference to Table 5 the PNAs used were 12m: PNA1878; 15: PNA1432; 14: PNA1835; 12: PNA1834; 11: PNA1877; 10: PNA1833; 9: PNA1876; 8: PNA1832; 7: PNA1875.

Figure 11.

LacZ expression and inhibition in *E. coli* with PNA, free peptide and a peptide-PNA conjugate. The values represent relative enzyme activities in *E. coli* K12 cultures grown in 10% LB broth. Antisense effects with 12 mer PNA 1834 (open circles), free peptide KFFKFFKFFK (SEQ ID NO: 1) (open squares), a mixture of free PNA and peptide (closed squares) and the peptide-PNA conjugate 1900 (closed circles).

Figure 12.

***E. coli* K12 growth and inhibition in MH broth by an anti-*acpP* peptide-PNA conjugate**. Cell growth is indicated by increased turbidity using OD_{550} measurements adjusted to a 1 cm path length. (A) *E. coli* K12 cells. (B) *E. coli* K12 cells with plasmid pKK223-3/*acpP-1*. Cultures were grown in the absence (closed circles) or presence (open squares) of added PNA (2 μM). The structure of the wild type chromosomal and mutant plasmid-borne copies of the *acpP* genes present in the two strains is shown, along with the antisense PNA, which is complementary only to the native *acpP* mRNA start codon region.

Figure 13.

Bactericidal antisense effects of an anti-*acpP* PNA against *E. coli acpP*. Cultures of *E. coli* K12 (open circles) and K12/*acpP-1* (closed circles) were established in MH broth at 10^5 cfu/ml and treated with PNA at 2 μM. The number of colony forming units was determined by plating.

Figure 14.

HeLa cell culture, noninvasive *E. coli* infection and antimicrobial PNA treatment. The images show HeLa cell cultures grown in MEMα, 10% FCS. The column at the left shows HeLa cell cultures without added *E. coli*, the center column shows HeLa cell cultures that were inoculated with *E. coli* K12, and the right column shows HeLa cell cultures that were inoculated with *E. coli* K12/*acpP-1*. The top panels of each column show cultures not

treated with PNA, and the rows below show cultures treated with increasing amounts of anti-*acpP* PNA. For cultures inoculated with *E. coli*, the colony forming units determined 24 hrs post inoculation are indicated.

Figure 15.
***B. subtilis* growth and inhibition by an anti-*acpA* peptidePNA.** Cell growth is indicated by increased turbidity at 550 nm. Culture were grown in the absence (closed circles) or presence of different concentration the anti-*acpA* PNA (open triangles with peak upwards, 0.5µM; closed diamonds, 1µM; hatched squares, 2µM; closed triangles, 3µM; open triangles with peak downwards, 4µM; open squares, 5µM).

Figure 16.
Bactericidal antisense effects of an anti-*acpA* PNA against *B.subtilis*. Cultures of *B.subtilis* were established at 10^6 CFU/ml in the absence (closed circles) or in presence (open circles) of anti-*acpP* peptide-PNA at 5 µM. The number of colony forming units (CFU) was determined by plating.

Examples

Materials and Methods

Bacterial strains

The permeable *E. coli* strain AS19 (Nikaido, 1994) was obtained from S. Pedersen (Univ. Copenhagen), *E. coli* strain DH5α was from Life Technologies (Gaithersburg, USA) and wild type *E. coli* strain K12 was from the *E. coli* Genetic Stock Center (Yale University).

Peptides and PNA

The peptide nucleic acids used in this study were synthesized as described (Christensen 1995). Briefly, peptide-PNA conjugates were synthesized by conventional maleimide coupling chemistry as will be reported in detail elsewhere. Briefly, PNA (1 mg, 0.29 μmol (1 equivalent)) was dissolved in minimal volume (typically 2 ml) of NMP: DMSO (8:2, v/v). SMCC maleimide reagent (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate; Pierce) (0.5 mg, 5 equivalents) and DIEA (40 μL) were added to PNA solutions and this was shaken gently at room temperature for 2.5 hr. The PNA-maleimide conjugates were precipitated by addition of excess dry cold ether (10 ml) and washed with ether containing 10% NMP (5 x 10 ml) in order to remove excess SMCC. The PNA-maleimide conjugate was dried under nitrogen, dissolved in 200 μL of DMF and allowed to react with the cysteine reduced peptide (3.25 mg, 5 equivalents) overnight. The conjugates were purified by HPLC (C_{18} column) using acetonitrile-H_2O containing 0.1% TFA as eluent and further characteized by MALDI-TOF mass spectrometry.

Reporter gene expression and inhibition by PNA

E. coli cultures were initiated with 10^4 cfu added to 100 μl of 10% LB broth in 1.5 ml siliconized microdilution tubes. The *lacZ* gene was induced with 50 μM isopropyl β-D-thiogalactopyranoside (IPTG). After overnight growth at 37°C, LacZ activity was assayed as described (Miller, 1972). Luciferase gene expression and inhibition was assayed using *E. coli* K12 carrying pBestluc and the Luciferase Assay System (Promega, cat no E1500).

Growth curves

E. coli K12 cells were inoculated at 10^5 cfu/ml in 100 μl of MH broth in a low binding 96 well microtiter plate (Costar 7424) with PNAs included. Plates were incubated at 37°C in a Molecular Dynamics Spectromax spectrophotometer set to shake the plate for 5 seconds and record turbidity at 550 nm at 5-minute intervals.

The minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for PNAs targeted to the particular binding sites were determined by monitoring the growth of *E. coli* K12 in the presence of a range of PNA concentrations. The lowest concentration that prevented growth by visual

inspection after overnight (16h - 24h as indicated) incubation from an inoculum of 10^5 cfu/ml was taken as the MIC. The growth media varied from experiment to experiment. Most MIC experiments were performed in LB broth diluted to 10% of normal strength; however, a few experiments were performed in full strength Mueller Hinton (MH) broth or Minimal Essential Media alpha supplemented with 10% fetal calf serum as indicated. The highest concentrations of PNA tested varied from experiment to experiment and are indicated.

Viable cell counts

E. coli cells in logarithmic growth were diluted to 10^5 cfu/ml and incubated with added PNA at 37°C in Costar 7424 microtitre plate wells. Samples were taken at time intervals, plated onto LB agar and grown at 37°C overnight and counted.

Transformation

Electrocompetent DH5α were prepared as described (Sambrook et al. 1989). The electrocompetent DH5α (35 μl) were transformed with 1 μl of pKK223-3/*acpP*-1 (30 ng/ml) using a BioRad GenePulser II, Hercules, CA, USA. The sample was electroporated in a 1 mm gap width cuvette at 1.8 kV, 25 mF, 200 ohms for 2.5 ms and the cells were left to recover in 250 μl of SOC-media at 37°C for 40 minutes.

For further growth experiments in liquid media, the bacteria were diluted to a concentration of 1.25 x 10^5 cells/ml in 10% LB low salt. Aliquots of 80 μl (~10^4 cells) were added to the wells of an "Ultra low attachment 96 well plate with lid", along with 20 μl of PNA SP4 (1.0 μM final concentration). The plate was incubated under the same conditions as described above.

Statistics

Values shown in the graphs are the mean of three replicates. Relative values were calculated using values obtained for cultures lacking PNA. The standard deviations are indicated.

Electrocompetent DH5α were prepared as described (Sambrook et al. 1989) and transformed with 1 μl of pKK223-3/*acpP-1* (30 ng/ml) and the cells were left to recover in 250 μl of "SOC"-media at 37°C for 40 minutes, as described (Sambrook et al. 1989). The bacteria were diluted to a concentration of 1.25 x 10^5 cells/ml in 10% LB low salt (5 g of NaCl/l). Aliquots of 80 μl (~10^4 cells) were added to the wells of an "Ultra low attachment 96 well plate" (Costar), along with 20 μl of PNA SP4 (1.0 μM final concentration). The plate was incubated under the same conditions as described above.

Example 1. Evaluating the size/activity relationship for antisense PNAs directed against the LacZ gene of *E. coli*.

Antisense PNAs are larger than most drugs and PNA size or length is likely to be an important parameter for efficiency. To evaluate the size/activity relationship for antisense PNAs in *E. coli* cultures, PNAs targeted to the start codon region of the chromosomal β-galactosidase gene (*lacZ*) (see Figure 9 and Table 5) were synthesized over the 7 to 15 mer size range as described in the Materials and Methods section.

Inhibition of *lacZ* expression was determined using the *E. coli* liquid culture assay described in the Materials and Methods section and in Good 1998a, Good 1998b. The results are illustrated in Table 5 and Figure 10 for a wild type *E. coli* strain (K12) and a permeable mutant (AS19) (Sekigushi 1967).

In both strains, PNAs in the 9 - 12 mer range were most active. The 7 and 8 mer PNAs tested were the least active. Also, truncated versions of the triplex-forming, anti-ribosomal RNA PNA were tested, and the shorter versions were not found to increase potency significantly in this case (Table 5: PNAs 1406, 1407 & 1410 versus PNA 1143). These results are consistent with reports of impressive antisense effects with even shorter oligomers (Wagner 1996). One would indeed expect an optimum PNA length because binding affinity to the RNA target increases with length, whereas uptake efficiency is expected to decrease dramatically with length. Also, shorter oligomers naturally offer higher sequence specificity at the expense of gene target specificity. Finally, as an initial test of selectivity, a mismatched 12 mer PNA (PNA 1878 versus PNA 1834) was tested and found to be significantly less active than the fully matched version (Table 5; Figure 10).

Example 2. Peptide-mediated delivery of antisense PNAs

The *E. coli* outer cell wall is a major barrier to PNA (Good 2000). Recently the synthetic peptide (KFFKFFKFFK (SEQ ID NO: 1)) was shown by Vaara (1996) to improve the permeability. To test whether this cell wall permeating peptide, when conjugated to PNA oligomers, could enhance the uptake and thus efficacy of antisense PNAs, the 12 and 15 mer anti-*lacZ* PNAs were synthesized with the KFFKFFKFFK (SEQ ID NO: 1) peptide attached via a flexible ethylene glycol-lysine linker (eg1) (AEEA-OH spacer Cat# GEN063030 AppliedBiosystems, Framingham, MA, USA) as described in the Materials and Methods section and in Table 5.

Various amounts of PNAs were then added to cultures of *E. coli* K 12 cultured in 10% LB Broth (Ausubel 2000) and the IC_{50} values were found by establishing the concentrations of PNAs causing a 50% reduction in β-galactosidase (*lacZ*) or luciferase (*luc*) expression in *E.*

coli K12. Similarly minimum inhibitory concentrations (MIC) were found by establishing lowest PNA concentrations that prevented *E. coli* growth by visual inspection after overnight (16h) growth from an inoculum of 10^5 cfu/ml. The maximum substance concentrations used were 3 μM for the anti-*lacZ* related PNAs and 5 μM for the anti-α-sarcin and *acpP* related PNAs.

Both the 12 and 15 mer conjugates were 15-20 fold more potent than the corresponding PNAs without attached peptides (Table 5; Figure 11). Also, consistent with the length comparison experiment (Example 1), the 12-mer peptide-PNA conjugate was significantly more active than the 15-mer version. Furthermore, the free peptide did not inhibit *lacZ* production (*e.g.* via general toxicity), nor did a mixture of free peptide and naked PNA. Thus covalent attachment of PNA to the carrier peptide was needed for efficient antisense effects on *E. coli* cells (Table 5; Figure 11).

To evaluate the specificity of the more potent anti-*lacZ* peptide-PNAs, one 15 mer (SP183 versus 1873) and two 12-mer mismatched peptide-PNA conjugates (1901 & SP182 versus 1900) were included in the study. These control constructs showed poor *lacZ* inhibition when compared to the fully matched constructs of similar size and composition. Also the anti-*lacZ* constructs showed very low inhibition of a second reporter gene (luciferase) and low general toxicity (Table 5). Therefore, the peptide-PNAs retain good overall specificity as PNAs.

To study the effect of the linker connecting the peptide and the PNA, peptide-PNA conjugates were assembled via maleimide SMCC coupling (PNA 1978) and the ethylene glycol linker (PNA 1900). As seen in Table 5, a peptide-PNA conjugate with the SMCC linker is more than 10 fold less potent than one with the ethylene glycol linked PNA (PNA 1900).

Also, peptides other than the KFFKFFKFFK (SEQ ID NO: 1) motif are able to carry PNA oligomers into bacteria as indicated by the comparable potencies for PNAs 1978, p29, p30, p31 and p32 (Table 5).

Example 3. Peptide-PNA conjugate inhibition of bacterial growth.

It has previously been shown that a triplex-forming PNA oligomer (PNA 1143) targeted to the α-sarcin loop of the 23S ribosomal RNA inhibits the growth of the permable *E. coli* strain AS19 (Good 1998b). To test whether attached peptides can also improve the potency of such anti-microbial PNAs, the KFFKFFKFFK (SEQ ID NO: 1) peptide was conjugated to a PNA with the nucleobase sequence H-jtjtjjt—tcctctc-NH_2 (the amino terminus (H-) corresponds to the 5'-end of a conventional oligonucleotide) as described in

the Materials and Methods section. The effect on bacterial growth of the resulting peptide-PNA conjugate (PNA 1934) as well as 4 "non-conjugated" versions were tested.

Minimum inhibitory concentrations (MICs) of the resulting peptide-PNA conjugate (PNA1934) as well as 4 "non-conjugated" versions (PNA 1143, 1410, 1406 and 1407; see Table 5) were found by establishing the lowest PNA concentrations that prevented *E. coli* growth by visual inspection after overnight (16h) growth from an inoculum of 10^5 cfu/ml in 10% LB broth. The MIC of PNA1934 was also established in Mueller Hinton broth (Cat# M9677, Sigma Chemical Co. St. Louis, MO. USA). The maximum concentrations of anti-α-sarcin related PNAs were 5 μM.

As seen in Table 5, the peptide-PNA conjugate (PNA1934) exhibited an MIC of 3 μM against *E. coli* K12 grown in Mueller Hinton broth and an MIC of 700 nM when grown in diluted LB broth (Table 5). Free peptide and PNA 1143 were not inhibitory at these concentrations, showing that conjugation of the peptide with PNA is needed for potent growth inhibitory effects of "non-permeable" *E. coli*.

Example 4. Peptide-PNA conjugate targeted against the essential *acpP* gene.

To test the potential for PNAs targeted against mRNA to provide antibacterial effects, a scan for antisense susceptible sites within the *E. coli* essential *acpP* gene was initially performed.

Fourteen peptide-PNA conjugates (see Table 1 below) were synthesised as described in the Materials and Methods section.

Table 1. Peptide PNA-conjugates targeted to *acpP*

peptide-PNA	Structure	SEQ ID NO:
SP4	H-KFFKFFKFFK-eg1-ctcatactct-NH_2	20
SP135	H-KFFKFFKFFK-eg1-cctatcaaaact-NH_2	
SP136	H-KFFKFFKFFK-eg1-actcttaaattt-NH_2	
SP137	H-KFFKFFKFFK-eg1-gatagtgctcat-NH_2	
SP138	H-KFFKFFKFFK-eg1-aacgcgttcttc-NH_2	
SP139	H-KFFKFFKFFK-eg1-gataattttctt-NH_2	
SP140	H-KFFKFFKFFK-eg1-ctgttcgccgat-NH_2	
SP141	H-KFFKFFKFFK-eg1-agtgctcatact-NH_2	
SP142	H-KFFKFFKFFK-eg1-gcgttcttcgat-NH_2	
SP143	H-KFFKFFKFFK-eg1-aattttcttaac-NH_2	
SP144	H-KFFKFFKFFK-eg1-cttaacgcgttc-NH_2	
SP145	H-KFFKFFKFFK-eg1-tcttcgatagtg-NH_2	
SP146	H-KFFKFFKFFK-eg1-tgctcatactct-NH_2	
SP147	H-KFFKFFKFFK-eg1-catactcttaaa-NH_2	

Growth conditions and PNA treatment: *E. coli* K12 was pre-grown overnight in 2 ml of 10% LB low salt media (Cat#L1703, Saveen, Malmö, SE (note, this is not standard LB) at 37°C with constant shaking at 225 rpm. Optical density was measured at 550 nm the following day and the culture was diluted to 1 x10^5 bacteria per ml in Mueller Hinton broth (Cat#L1703, Saveen, Malmö, SE). Aliquots (80 µl) corresponding to approximately 10^4 cells were added to the wells of an "Ultra low attachment 96 well plate with lid" (COSTAR®, Corning Inc., NY). Fourteen peptide-PNA constructs were diluted in water and aliquots were transferred into microtitre plate wells to provide a PNA concentration ranging from 100 to 1000 nM and a final culture volume of 100 µl. The 96 well plate was incubated for 24 hours in a VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA) at 37°C with shaking and OD measurements each five minutes. Shaking time was set to five seconds and optical density was measured at 550 nm.

The minimum inhibitory concentrations (MICs) for peptide-PNAs targeted to different *acpP* mRNA binding sites were determined by monitoring the growth of *E. coli* K12 in the presence of a range of peptide-PNA concentrations. The lowest concentration that prevented growth after 24 hours of incubation was taken as the MIC. The highest concentrations tested were 10 µM of the test substance.

The peptide-PNA binding sites and the resulting MICs are illustrated in Figure 6. The results indicate that the start codon region including the Shine Delgarno sequence is

particularly susceptible to antisense inhibition. All but one of the peptide-PNAs targeting this region showed low MIC values, whereas peptide-PNAs targeting downstream sites were not inhibitory at the highest concentrations tested (10 μM). The results support the idea that the start codon region is a susceptible target sequence for antisense inhibition by peptide-PNAs. Also, the results suggest that the number of susceptible binding sites within mRNA is restricted and this improves the probabilities for gene specific effects.

The peptide-PNA conjugate showing the lowest MIC, the SP4, showed an MIC against *E. coli* K12 of 1 μM in rich broth and an even lower MIC in dilute broth (Figure 6, Figure 12A, Table 5).

Example 5. Bacteriocidal effect of peptide-PNA conjugate directed against *acpP*

To test the potential for PNAs targeted against mRNA to provide antibacterial effects, the SP4 PNA complementary to the start codon region of the essential *acpP* gene was tested for its bacteriocidal effects and its specificity.

Initially the essential *E. coli acpP* gene encoding the acetyl carrier protein Acp was cloned and selected bases were altered to eliminate the potential binding of SP4 PNA while still encoding native Acp.

The bacteriostatic and bacteriocidal effects of the anti-*acpP* peptide-PNA (SP4) were demonstrated in two ways. First, the MICs of SP4 and a control peptide-PNA with a scrambled PNA sequence (SP181, SEQ ID NO: 51) were determined as described in the Materials and Methods section. The control peptide-PNA had a 15 fold higher MIC than the fully matched version SP4 (Table 5). This result is consistent with an *acpP* specific inhibitory effect for SP4. Second, to confirm that growth inhibition was due to down regulation of *acpP* expression, the *E. coli acpP* gene was cloned and selected bases altered to eliminate the potential for PNA binding, but still encode native Acp as described.

Primers complementary to each end of the *acpP* gene were designed and ordered from Interactiva Biotechnologie (Ulm, Germany). The *E. coli acpP* gene was amplified by PCR using the primers, 5′-GAGAATTCATGAGCACTATCGAAGAAC-3′ (SEQ ID NO: 15) and 5′-AGTTAAGCTTGACCGCCTGGAGATGTT -3′ (SEQ ID NO: 16). These primers contain sites for the restriction enzymes *Eco*R I and *Hind* III and are designed to mutagenise the bases between the Shine-Delgarno sequence and the AUG initiation codon (see Figure 2, 6 and 14).

A primer extension reaction was performed with 10 pmol of each of the primers, 0.2 mM dNTP mix, 2.5 U Taq polymerase (Amersham Pharmacia) and 1/40 of a "normal sized" colony of K12 cells (grown overnight on an LB plate at 37°C) in a total volume of 100 µl buffered with PCR-buffer (the above products were included in "Advantage® PCR Kit", Clonetech, USA). The reaction was incubated in a "Peltier Termal Cycler" (PTC-225, MJ Research, NY) with 1 minute at 94°C followed by 1 minute at 50°C and 1 minute at 70°C for 30 cycles. The amplified sample was run on a 1.5% agarose gel for 1 hour. The gel was stained with ethidium bromide and the bands were visualised with long UV light. A fragment corresponding in size to the *acpP* gene (275 nt) was extracted and cleaved with 0.5 µl each of the enzymes *Eco*R I and *Hind* III (New England Biolabs, USA), and ligated into the expression vector pKK223-3 (Amersham-Pharmacia, Uppsala, SE). A map of the resulting construct is shown in Figure 5.

Transformation of *E. coli* cells (see Materials and Methods section) with this target-site-altered version of *acpP* - called *acpP*-1 - rescued the bacteria from PNA growth inhibition (Figure 12B). Complementation by the altered *acpP* allele gives strong genetic evidence for specificity and confirms an antisense mechanism of action for the anti-*acpP* peptide-PNA.

The antibacterial effects of peptide-PNA conjugates on *E. coli* cells were examined by assessing cell viability as described in the Materials and Methods section.

Within one hour of exposure to 2 µM anti-*acpP* PNA SP4, the number of colony forming units decreased dramatically from 10^5/ml and no viable cells were detected after three hours, showing that the PNA treatment was bactericidal (Figure 13). Again, the strain containing the binding-site-altered allele was resistant to PNA.

Example 6. Selection of *acpP*-1 containing transformed cells in a mixed culture.

To further characterise the antibacterial properties of the peptide-PNA conjugate (SP4) and evaluate its potential for selecting plasmid containing bacteria, a mixed culture of wild type E. coli and *E. coli* transformed with plasmid pKK223-3/*acpP-1* was set up and plasmid bearing bacteria were selected by adding SP4 to the culture. The experiment is illustrated in Fig. 3.

Briefly, *E. coli* K12 and K12/pKK223-3/*acpP-1* were pre-grown overnight in 2 ml of 10% LB low salt media at 37°C with constant shaking at 225 rpm. Optical density was measured at 550 nm the following day and the cultures were diluted to 2.5 x 10^5 bacteria per ml in 10% Luria Bertani broth. Aliquots (40 µl) corresponding to approximately 10^4 cells of mono or

mixed cell types were added to the wells of an "Ultra low attachment 96 well plate with lid" (COSTAR®, Corning Inc., NY). PNA treated samples included 20 µl of PNA SP4 (1.0 µM final concentration), and 40 µl of 10% LB low salt were added where needed to make a total culture volume of 100 µl. The 96 well plate was incubated for 24 hours in a VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA) at 37°C with shaking and OD measurements each five minutes. Shaking time was set to five seconds and optical density was measured at 550 nm.

Samples of 2 µl were harvested from the mono, mixed and transformed cell cultures after 0, 1, 2, 3 and 24 hours. The harvested samples were mixed with 98 µl of water, and 50 µl of each mixture were plated out on standard Luria-Bertani plates (Sambrook et al. 1989). The remaining diluted cultures were used to make further dilutions that were also spread out on LB-plates. The plates were incubated at 37°C for 48 hours. To test for Amp^r, colonies derived from each harvest time as many as possible and over one hundred were picked and transferred individually to LB-plates containing ampicillin (75 µg/ml). The LBA-plates were incubated at 37°C for 24 hours and the number of growing colonies was counted relative to the total number of colonies transferred.

The result of this experiment is presented in Table 2 below.

Table 2: Selection for *acpP-1* strain from a mixed culture

			PNA 1 µM		
	K12	K12/*acpP-1*	K12	K12/*acpP-1*	K12+ K12/*acpP-1*
time	cfu	cfu	cfu	cfu	cfu/%Amp^r
0 h	265	192	136	217	224/~50
1 h	152	101	3	55	24/83.3
2 h	73	22	1	29	12/100
3 h	695	80	0	81	21/100
24 h	$>10^4$	$>10^4$	0	$>10^4$	$>10^4$/100

The table shows the growth of wild type and pKK223-3/*acpP-1* carrying (K12/*acpP-1*) *E. coli* K12 on LB plates.The cells were pre-grown for different times as mono or mixed cultures in the presence or absence of 1 µM PNA SP4, as indicated. As pKK223-3/*acpP-1* not only carries a PNA SP4 target site altered copy of the *acpP* gene, but also the ampicillin resistance gene, the amount of plasmid carrying colonies in the mixed culture was deduced by colony transfer to LB-Amp plates. The amount of Amp^r clones is seen in the right-most column.

Conclusion

The results of this study show that antisense PNA can be used to select for specific strains from a mixed *E. coli* culture when targeted against an essential gene.

Example 7. Selection of *acpP*-1 transformants following electroporation.

To stydy if the antibiotic-free PNA-based selection procedure was sufficiently efficient to allow for selection of transformed cells after a standard transformation procedure (electrotransformation), the following experiment was performed.

Electrocompetent DH5α were prepared and transformed with 1 μl of pKK223-3/*acpP-1* (30 ng/ml) as described in the Materials and Methods section.

Then the bacteria were diluted to a concentration of 1.25 x 10^5 cells/ml in 10% LB low salt (5 g NaCl/l). Aliquots of 80 μl (~10^4 cells) were added to the wells of an "Ultra low attachment 96 well plate" (Costar), along with 20 μl of PNA SP4 (1.0 μM final concentration) and incubated in a VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA) at 37°C with shaking and OD measurements each five minutes. Shaking time was set to five seconds and optical density was measured at 550 nm.

Samples of 2 μl were harvested from the electrotransformed cell cultures after 0, 2, 4, 6, 18, and 24 hours. The harvested samples were mixed with 98 μl of water, and 50 μl of each mixture were plated out on standard Luria-Bertani plates (Sambrook et al. 1989). The remaining diluted cultures were used to make further dilutions that were also spread out on LB-plates. The plates were incubated at 37°C for 48 hours. To test for Amp^r, colonies derived from each harvest time as many as possible and over one hundred were picked and transferred individually to LB-plates containing ampicillin (75 μg/ml). The LBA-plates were incubated at 37°C for 24 hours and the number of growing colonies was counted relative to the total number of colonies transferred.

The result is presented in Table 3 below.

Table 3: Selection for *acpP*-1 transformants

time	transformed_DH5α	
	cfu	% Amp^r
0 h	-	1.8
2 h	10	40.0
4 h	14	71.4
6 h	32	96.9
18 h	$>10^4$	100
24 h	$>10^4$	100

The table shows the number of colony forming units (cfu) in a culture of DH5α cells transformed with pKK223-3/*acpP*-1 and grown in the presence of 1 μM PNA SP4 for different times. The right-hand column shows the amount of Amp^r cells.

To determine whether the Amp^r colonies carried pKK223-3/*acpP-1,* a sample population of colonies that arose after electroporation and PNA treatment was picked and grown in 2 ml of LB with ampicillin overnight at 37°C with shaking (225 rpm). Plasmids were purified using GFX Micro Plasmid-prep Kit (Amersham Pharmacia, Uppsala, SE) fractionated in a 1% agarose/Tris-borate-acetate gel electrophoresis, stained with ethidium bromide (0.5 μg/ml) and photographed.

The resulting photograph is shown in Figure 4. As seen in Figure 4, all the tested 11 SP4 resistant *E. coli* cultures contain plasmids of a size indistinguishable from the size of the pKK223-3/*acpP*-1 control plasmid prepared by conventional Amp^r selection.

Example 8. Antisense peptide-PNA inhibition of reporter gene expression in *B. subtilis*

To test whether antisense peptide-PNAs can control reporter gene expression and thus be used for selection in the gram positive bacterium *B. subtilis*, a wildtype (168) strain of Bacilus was constructed which expresses an enhanced version of the reporter green fluorescence protein (GFP) gene and these cells were treated with antisense peptide-PNAs.

Bacterial strains and PNA

B. subtilis wild type strain 168 was obtained from the Institute Pasteur strain collection, France. The PNA was synthesised as described in the Materials and Methods section. Luria-Bertani medium was used for growing and manipulating *E. coli* and *B. subtilis*. Cultures

were grown at 37°C on a rotary shaker (222 r.p.m.) overnight. Antibiotics were used at the following concentrations: tetracycline, 10 μg/ml; ampicillin 75 μg/ml.

Table 4. Plasmids and PNAs used in this study

Strain or plasmid	Description	Source
Plasmids		
pSPT245	Shuttle vector pRN8054::pSP64	Morfeldt et al.(1996)
pAS1	pSPT245 containing the xylR gene and the xylA promoter	Tegmark et al.(2000)
pSPT245-GFP+	pSPT245 containing the GFP *gfp* indicator gene	This study
pSPT245-GFP+-Pxyl	pSPT245-GFP+ containing *gfp* indicator gene under the control of the xylA promoter	This study
peptide-PNAs		
1900	H-KFFKFFKFFK-eg1-catagctgtttc-NH_2	
1901	H-KFFKFFKFFK-eg1-caatgtcgtttc-NH_2	

Construction of enhanced GFP reporter vector.

Plasmids used are listed in Table 4. All plasmid constructs were first propagated in *E. coli* DH5α and then transferred to *B. subtilis* 168. Competent *E.coli* cells were prepared and transformed by the method of Sambrook et al. (1982). *B. subtilis* was transformed by electroporation as described below. *E. coli* transformants were selected on LB (Difco, LePont de Claix, France) plates containing 75 μg of ampicillin ml^{-1} and *B. subtilis* transformants on LB agar plates containing 10 μg of tetracycline ml^{-1}. To optimize expression of *gfp* in gram positive bacteria we introduced a consensus Shine-Delgarno sequence GGAGG just upstream of the start codon. The plasmid pEGFP was used as template; this plasmid contains a *gfp* derivative with enhanced fluorescence properties (Clonetech, Palo Alto, CA). Also, to enable comparison with previous antisense PNA experiments with *E. coli,* the target site for peptide-PNA 1900 was introduced at the start codon region. These new sequences were included in the upstream primer for a PCR reaction, where an ~800-bp fragment encompassing the *gfp* gene preceded by the consensus RBS and PNA target site with the following primers containing restriction sites: 5'aaactgcaG(*Pst*I)GAG*Gaaacagctatg*ctcgag(XhoI)-atatcgaagggcgaggag (SEQ ID NO: 17) (the fragment containing the Staph. RBS and PNA's target site is in italics) and 5'gatgagctct(*Sac*I)cggctcgatgatcc (SEQ ID NO: 18). The *gpf* gene was amplified with *T. aquaticus* DNA polymerase (Amersham Pharmacia), using a PCR recaction consisting of 95°C for 30 sec., 55°C for 1 min and 72°C for 1 min which was repeated 30 times. The fragment was first cut with SacI and analysed in 1% agarose gel. The cleaved ~800-bp

PCR fragment was gel purified and ligated to the SacI/SmaI site of the polylinker region of the *E. coli* gram positive shuttle vector pSPT245. The recombinant plasmid containing the promoterless *gfp* reporter gene, designated pSPT245-GFP, was amplified in *E. coli* and verified by restriction analysis. Plasmid DNA was extracted by using the GFX Micro Plasmid Preo Kit (Amersham Pharmacia Biotech Inc,USA). Plasmid pSPT245-GFP+-Pxyl, carrying the *gfp* gene under the control of the xylose promoter, was constructed by cloning a SphI/BamHI fragment containing the entire xylR gene and xylA promoter region from pAS1 into pSPT245-GFP cut with *Sph*I and *Bam*HI.

Transformation of *B. subtilis*

An overnight culture of the recipient cells was grown in TSB broth with constant aeration at 37ºC and diluted 1/20 into 40 ml of fresh B2 broth in a 500 ml Klett flask. The culture was grown with constant aeration at 37°C for 2h and harvested by centrifugation. The cells were washed once in an equal volume of deionized water. Then the competent cells were washed once in 0.15 M NaCl and suspended in 500 µl of 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-maleate buffer (pH 7.0) containing 0.1 M $CaCl_2$.

The electrocompetent cells were mixed with an equal volume of 0.5 M sucrose and incubated 5 min at room temperature. For electroporation 50 µl of cell suspension was incubated with 1.5 µl of DNA for 30 min at room temperature. Then the sample was transferred to a 1 mm electroporation cuvette and pulsed once at 2 kV with a 25 µF capacitor (10 kV/cm field strength for 2.5 ms) in a BioRad GenePulser II, Hercules, CA, USA. The sample was immediately resuspended in 950 µl of TSB broth and transferred to a 2.5 ml tube and shaken for 1.5 h at 225 rpm at 37°C. The cells were then plated on LB agar plates containing the appropriate selective agent and incubated at 37°C for 24-48h.

B. subtilis growth and antisense PNA treatment: *B. subtilis* cells were pre-grown overnight in 2 ml of Mueller Hinton broth at 37°C with constant shaking at 225 rpm, with 5 mg/ml tetracyline. Optical density was measured at 550 nm the following day and the cultures were diluted to 2.5×10^5 bacteria per ml in 10% Luria Bertani broth. Aliquots (40 µl) corresponding to approximately 10^4 cells of mono or mixed cell types were added to the wells of an "Ultra low attachment 96 well plate with lid" (COSTAR®, Corning Inc., NY). PNA treated samples included 20 µl of PNA SP4 (1.0 µM final concentration), and 40 µl of Mueller Hinton broth were added where needed to make a total culture volume of 100 µl. The 96 well plate was incubated for 24 hours in a VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA) at 37°C with shaking and OD measurements each five minutes. Shaking time was set to five seconds and optical density was measured at 550 nm.

Fluorescence Microscopy and image analysis

Following PNA treatment, *B. subtilis* cells were collected by centrifugation at 5000 rpm for 5 min. The cell pellets were resuspended in 1 X PBS buffer and fluorescence microscopy was performed using a Leica DMRXA microscope with a cooled frame CCD camera using excitation and emission wavelengths of 488 and 507 nm for GFP and 490 and 520 nm for FITC, with Openlab™ image analysis and presentation.

Results

The expression vector was constructed to provide a binding-site for the peptide-PNA 1900 (Figure 7). This PNA was shown previously to provide potent antisense inhibition in *E. coli* (Good et al. 2001). The transformed version of *B. subtilis* showed strong green fluorescence when visualised by fluorescence microscopy and the parent strain showed very low background fluorescence (Figure 8). For cultures of *B. subtilis* grown in the presence of antisense peptide-PNA 1900, the cells grew at a normal rate and appeared normal when viewed under standard white light conditions. When viewed using fluorescence microscopy, the visible green fluorescence was markedly or entirely reduced by the peptide-PNA 1900 (Figure 8). A control peptide-PNA (1901) did not reduce visible green fluorescence. Therefore, it appears that the antisense peptide-PNA entered *B. subtilis* cells and inhibited *gfp* expression by binding to *gfp* mRNA. The results suggest that antisense peptide-PNA inhibiton strategies can be extended to *B. subtilis* and possibly other gram positive bacteria.

Example 9. Using antisense PNA directed against the *acpP* gene to remove bacterial growth from a cell culture of human HeLa cells.

To evaluate any possible anti-*acpP* PNA (SP4) toxicity to eukaryotic cells as well as to examine the anti-bacterial potential of antisense inhibition in the presence of eukaryotic cells, the anti-*acpP* PNA was tested against *E. coli* grown in cell culture medium. Interestingly, the PNA is 10 fold more potent (MIC = 200 nM) in this medium than in MH broth (Table 5).

Briefly, HeLa cells were obtained from CloneTech, Palo Alto, CA, USA, Cat#C3001-1. HeLa cells were cultured in Minimal Essential Medium alpha (MEMα), 10% fetal calf serum (Life Technologies). Prior to infection, HeLa cells were suspended by scraping, transferred to 96 well Falcon 3872 plates and grown to approximately 30% confluence. Freshly grown *E. coli* K12 cells were added at 10^5 cfu/ml. Following infection, PNA solution or an equivalent volume of water was added and the plates were incubated overnight at 37°C in 5% CO_2.

The PNA did not visibly affect HeLa cell growth at the highest concentration tested (20 μM) (Figure 14), and PNA at 2 μM or higher appeared to fully cure the HeLa culture of the infection. No live bacteria were detected from these samples in an agar colony forming unit test, where dilutions were spread on LB plates, incubated overnight at 37°C and the number of colony forming unit detected was used to calculate the value for cfus/ml in the sample, confirming that the anti-*acpP* PNA is bactericidal under these conditions. Again, the sequence specificity of the effect was evaluated in cultures infected with *E. coli* carrying the binding-site-altered *acpP* allele (K12-*acpP*-1) and this infection was resistant to PNA up to at least 20 μM (Figure 14).

Example 10. Bactericidal antisense PNA effects against *B. subtilis*.

To indicate whether the antisense strain selection approach could have any potential in other microbial species. We targeted the *acpA* gene in *B. subtilis*, which is a gram positive bacterium often used in industrial fermentations.

Bacterial strain and PNA: *Bacillus subtilis* wild type strain 168 was obtained from the Institute Pasteur strain collection, France. The PNA was synthesised as described in the Materials and Methods section.

Culture: Cultures were pre-grown overnight in 2 ml 50% Mueller-Hinton broth at 37°C with constant shaking at 225 rpm. Optical density was measured at 550 nm the following

day and the cultures were diluted to $2.5 \times 10^{4-5}$ bacteria per ml. Aliquots (40 µl) corresponding to approximately 10^4 cells of mono or mixed cell types were added to the wells of an "Ultra low attachment 96 well plate with lid" (COSTAR®, Corning Inc., NY). PNA treated samples included 20 µl PNA (1.0 µM final concentration), and medium was added where needed to make a total culture volume of 100 µl. The 96 well plates were incubated in a VERSAmax spectrophotometer (Molecular Devices Corporation, CA, USA) at 37°C with shaking and OD measurements each three minutes. Shaking time was set to fifteen seconds and optical density was measured at 550 nm.

Viable cell counts: *B. subtilis* grown to logarithmic growth were diluted to 10^6 CFU/ml and incubated with added PNA. Samples were taken at time intervals, plated onto LB agar and grown at 37°C overnight.

Results: Although not previously shown to be an essential gene, the *B. subtilis* ACP sequence is 57.1% identical to that of *E. coli* ACP. To resemble the design useful in *E. coli*, we designed an antisense peptide-PNA against the *B. subtilis acp.* This anti-*acpA* peptide-PNA conjugate (H-KFFKFFKFFK-egl-tgccatagca-NH_2 (SEQ ID NO: 52)) showed inhibition of bacterial growth *B. subtilis* (Fig. 15), similar to the effects seen in *E. coli*.

The antibacterial effects of the anti-*acpA* peptide-PNA conjugate on *B. subtilis* cells were further examined by assessing cell viability. When freshly grown cells were treated with 5 µM peptide-PNA, the number of colony-forming unit (CFU) rapidly decreased from 10^6CFU/ml to the extent that no viable cells were detected after 3 h (Fig. 16). The results show that the anti-*acpP* peptide-PNA was bactericidal against *B. subtilis*.

Conclusion

The results show that antisense PNA against *Bacillus subtilis acpP* gene is bactericidal and open the possibilities to use antisense PNAs as a substitute for antibiotics for strain selection and construction in research and for industrial applications.

Table 5. PNAs and antisense control of *E. coli* K12 gene expression and growth

PNA designation and sequence		# PNA bases	IC_{50} *lacZ* (μM)	IC_{50} *luc* (μM)	MIC (μM)
anti-*lacZ* PNAs, peptide-PNAs & free peptides					
1432	H-ggtcatagctgtttc-lysNH_2	15	2	>3	>3
1835	H-gtcatagctgtttc-lysNH_2	14	1	>3	>3
1834	H-catagctgtttc-lysNH_2	12	0.9	>3	>3
1877	H-atagctgtttc-lysNH_2	11	0.7	>3	>3
1833	H-tagctgtttc-lysNH_2	10	0.8	>3	>3
1876	H-agctgtttc-lysNH_2	9	0.9	>3	>3
1832	H-gctgtttc-lysNH_2	8	1	>3	>3
1875	H-ctgtttc-lysNH_2	7	1	>3	>3
1878	H-caatgtcgtttc-lysNH_2 (mismatched 1834)	12	3	>3	>3
111	H-cccctattgtcc-lysNH_2 (unrelated control)	12	3	>3	>3
1176	H-gcaagcgactgtgga-lysNH_2 (unrelated control)	15	>3	>3	>3
1873	H-KFFKFFKFFK-eg1-ggtcatagctgtttc-NH_2	15	0.3	>3	>3
SP183	H-KFFKFFKFFK-eg1-gtgactcgatgtctt-NH_2 (scrambled 1873)	15	2	>3	>3
1900	H-KFFKFFKFFK-eg1-catagctgtttc-NH_2	12	0.05	>3	>3
1901	H-KFFKFFKFFK-eg1-caatgtcgtttc-NH_2 (mismatched 1900)	12	1	>3	>3
SP182	H-KFFKFFKFFK-eg1-acatgtcgtctt-NH_2 (scrambled 1900)	12	2	2	2
1978	H-KFFKFFKFFKC-smcc-eg1-catagctgtttc-NH_2[a]	12	0.2	3	>3
p29	H-IKFLKFLKFLC-smcc-eg1-catagctgtttc-NH_2	12	0.2	2	>3
p30	H-VDKGSYLPRPTPPRPIYNC-smcc-eg1-catagctgtttc-NH_2	12	0.4	2	2
p31	H-ILPWKWPWWPWRRGC-smcc-eg1-catagctgtttc-NH_2	12	0.4	>3	>3
p32	H-KLAKALKKLLC-smcc-eg1-catagctgtttc-NH_2	12	0.3	>3	>3
	H-KFFKFFKFFK-NH_2		>3	>3	>3
anti-α-sarcin rRNA PNAs & peptide-PNAs					
1143	H-jtjtjjt-(eg1)$_3$-tcctctc- lysNH_2	14			5
1410	H-tjtjjt-(eg1)$_3$-tcctctc- lysNH_2	13			4
1406	H-jtjjt-(eg1)$_3$-tcctctc- lysNH_2	12			4
1407	H-tjjt-(eg1)$_3$-tcctctc- lysNH_2	11			>5
1934	H-KFFKFFKFFK-eg1-jtjtjjt-(eg1)$_3$-tcctctc- lysNH_2	14			0.7 3[b]
anti-*acpP* peptide-PNAs					
SP4	H-KFFKFFKFFK-eg1-ctcatactct-NH_2	10			0.2 1[b] 0.2[c]
SP181	H-KFFKFFKFFK-eg1-tcactatctc-NH_2 (scrambled SP4)	10			3

Structures are shown in uppercase letters for peptide sequences and lower case letters for PNA sequences. The PNAs are written from their amino to carboxy termini, and the amino terminus corresponds to the 5'-end of a conventional oligonucleotide, note that -NH_2 indicate carboxyl-amide i.e. corresponds to the -COOH terminal of conventional peptides. The IC_{50} values indicate substance concentrations causing a 50% reduction in β-galactosidase (*lacZ*) or luciferase (*luc*) expression in *E. coli* K12. Minimal inhibitor concentrations (MIC) were the lowest PNA concentrations that prevented *E. coli* growth by visual inspection after overnight (16h) growth from an inoculum of 10^5 cfu/ml. The

maximum substance concentrations used were 3 μM for the anti-*lacZ* related PNAs and 5 μM for the anti-α-sarcin and *acpP* related PNAs. The J bases indicate pseudo-isocytosine. [a] smcc designates PNA-maleimide – cystein-peptide coupling via the smcc maleimide-active ester reagent. Cells were grown in LB broth diluted to 10% of normal strength, except where indicated as growth in [b] full strength Mueller Hinton (MH) broth or [c] Minimal Essential Media alpha, 10% fetal calf serum.

Table 6 Sequences used in the study

SEQ ID NO	SEQUENCE	PNA#
1	KFFKFFKFFK	
2	KFFKFFKFFKC	
3	IKFLKFLKFLC	
4	VDKGSYLPRPTPPRPIYNC	
5	ILPWKWPWWPWRRGC	
6	KLAKALKKLLC	
7	CTCATACTCT	
8	CATACTCTTAAA	
9	CCTATCAAAACT	
10	TGCTCATACTCT	
11	ACTCTTAAATTT	
12	AGTGCTCATACT	
13	TCTTCGATAGTG	
14	GATAGTGCTCAT	
15	GAGAATTCATGAGCACTATCGAAGAAC	
16	AGTTAAGCTTGACCGCCTGGAGATGTT	
17	AAACTGCAGGAGGAAACAGCTATGCTCGAGATATCGAAGGGCGAGGAG	
18	GATGAGCTCTCGGCTCGATGATCC	
19	AACAGAATTCATGAGCACTA	
20	H-KFFKFFKFFK-eg1-ctcatactct-NH_2	SP4
21	H-KFFKFFKFFK-eg1-jtjtjjt-$(eg1)_3$-tcctctc- lysNH_2	1934
22	...AUUUAAGAGUAUGAGCACUA....	
23	...AACAGAAUUCAUGAGCACUA....	
24	...GAAACAGCUAUGACCAU.......	
25	AGUUUUGAUAGGAAAUUUAAGAGUAUGAGCACUAUCGAAGAACGCGUUAAGAAAAUUAUCGGCGAACAG	
26	H-ggtcatagctgtttc-lysNH_2	1432
27	H-gtcatagctgtttc-lysNH_2	1835
28	H-catagctgtttc-lysNH_2	1834
29	H-atagctgtttc-lysNH_2	1877
30	H-tagctgtttc-lysNH_2	1833
31	H-agctgtttc-lysNH_2	1876

Table 6 Sequences used in the study, continued.

SEQ ID NO	SEQUENCE	PNA#
32	H-gctgtttc-lysNH_2	1832
33	H-ctgtttc-lysNH_2	1875
34	H-caatgtcgtttc-lysNH_2	1878
35	H-cccctattgtcc-lysNH_2	111
36	H-gcaagcgactgtgga-lysNH_2	1176
37	H-KFFKFFKFFK-eg1-ggtcatagctgtttc-NH_2	1873
38	H-KFFKFFKFFK-eg1-gtgactcgatgtctt-NH_2	SP183
39	H-KFFKFFKFFK-eg1-catagctgtttc-NH_2	1900
40	H-KFFKFFKFFK-eg1-caatgtcgtttc-NH_2	1901
41	H-KFFKFFKFFK-eg1-acatgtcgtctt-NH_2	SP182
42	H-KFFKFFKFFKC-smcc-eg1-catagctgtttc-NH_2	1978
43	H-IKFLKFLKFLC-smcc-eg1-catagctgtttc-NH_2	p29
44	H-VDKGSYLPRPTPPRPIYNC-smcc-eg1-catagctgtttc-NH_2	p30
45	H-ILPWKWPWWPWRRGC-smcc-eg1-catagctgtttc-NH_2	p31
46	H-KLAKALKKLLC-smcc-eg1-catagctgtttc-NH_2	p32
47	H-jtjtjjt-$(eg1)_3$-tcctctc- lysNH_2	1143
48	H-tjtjjt-$(eg1)_3$-tcctctc- lysNH_2	1410
49	H-jtjjt-$(eg1)_3$-tcctctc- lysNH_2	1406
50	H-tjjt-$(eg1)_3$-tcctctc- lysNH_2	1407
51	H-KFFKFFKFFK-eg1-tcactatctc-NH_2	SP181
52	H-KFFKFFKFFK-eg1-tgccatagca-NH_2	2316

References

Aldrian-Herrada, G. et al. A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. Nucleic. Acids Research 26, 4910-4916 (1998).

Ausubel, F. B., R Kingston, RE Moore DD Seidman, JG Smith, JA Struhl, K. (2000). Current protocols in molecular biology, John Wiley and Sons, Inc.

Christensen, L. et al. Solid-phase synthesis of peptide nucleic acids. J. Peptide Sci 1, 175-183 (1995).

Christensen, L., Fitzpatrick, R., Gildea, B., Petersen, K. H., Hansen, H. F., Koch, T., Egholm, M., Buchardt, O., Nielsen, P. E., Coull, J. & et al. (1995). Solid-phase synthesis of peptide nucleic acids. J Pept Sci 1(3), 175-83.

Cronan, J.E., Rock, C.O. (1996) in Escherichia coli and Salmlonella: cellular and molecular biology, Edn. 2nd 612-636 (American Society for Microbiology, Washington, D.C.).

Cutrona, G. et al. Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal. Nature Biotechnology 18 300-303 (2000)

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. Science 264(5157), 375-82.

Demidov, V.V. et al. Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. 48, 1310-1313 (1994).

Dervan PB, Burli RW.: Sequence-specific DNA recognition by polyamides. Curr Opin Chem Biol. 1999 Dec;3(6):688-93.

Egholm, M. et al. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365, 566-568 (1993).

Giesen, U. et al. A formula for thermal stability (Tm) prediction of PNA/DNA duplexes. Nucleic Acids Res. 26, 5004-5006 (1998).

Good, L. & Nielsen, P. E. (1997). Progress in developing PNA as a gene-targeted drug. Antisense Nucleic Acid Drug Dev 7(4), 431-7.

Good, L. & Nielsen, P. E. (1998a). Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. Nat Biotechnol 16(4), 355-8.

Good, L. & Nielsen, P. E. (1998b). Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. Proc Natl Acad Sci U S A 95(5), 2073-6.

Good, L. Awasthi, S.K, Dryselius, R., Larson, O. and Nielsen, P.E. (2001) Bactericidal antisense effects of peptide-PNA conjugates. Nature Biotechnology, in press.

Good, L., Sandberg, R., Larsson, O., Nielsen, P.E. & Wahlestedt, C. (2000).Antisense PNA effects in escherichia coli are limited by the outer- membrane LPS layer. Microbiology 146, 2665-2670.

Gottesfeld JM, Turner JM, Dervan PB.: Chemical approaches to control gene expression. Gene Expr. 2000;9(1-2):77-91

Hancock, R.E. (1997) Peptide antibiotics. Lancet 349, 418-422.

Hanvey, J.C. et al. Antisense and antigene properties of peptide nucleic acids. Science 258, 1481-1485 (1992).

Harth, G., Zamecnik, P.C., Tang, J.Y., Tabatadze, D. & Horwitz, M.A. Treatment of Mycobacterium tuberculosis with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication. Proc. Natl. Acad. Sci. U S A 97, 418-423 (2000).

Jensen, K.K., Orum, H., Nielsen, P.E. & Norden, B. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. Biochemistry 36, 5072-5077 (1997).

Knudsen, H. & Nielsen, P.E. Antisense properties of duplex- and triplex-forming PNAs. Nucleic Acids Research 24, 494-500 (1996).

Larsen, H. J., Bentin, T. & Nielsen, P. E. (1999). Antisense properties of peptide nucleic acid. Biochim Biophys Acta 1489(1), 159-66.

Miller, J.H. Experiments in Molecular Genetics. (Cold Spring Harbour Press, Cold Spring Harbour; 1972).

Morfeldt, E., Tegmark, K., Arvidson, S. (1996) Transcriptional control of the agr-dependent virulence gene regulator, RNAIII in Staphylococcus aureus Mol. Microbiol., 21: 1227-1237.

Murray, R.W., Schaadt, R.D., Zurenko, G.E. & Marotti, K.R. Ribosomes from an oxazolidinone-resistant mutant confer resistance to eperezolid in a Staphylococcus aureus cell-free transcription- translation assay. Antimicrob. Agents Chemother. 42, 947-950 (1998).

Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254(5037), 1497-500.

Nielsen, P.E., Haaima, G. Peptide nucleic acid (PNA). A DNA mimic with a pseudopeptide backbone. Chemical Society Reviews 96, 73-78 (1997).

Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264, 382-388 (1994).

Norton, J.C., Piatyszek, M.A., Wright, W.E., Shay, J.W. & Corey, D.R. Inhibition of human telomerase activity by peptide nucleic acids. Nature Biotechnol. 14, 615-619 (1996).

Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J. X., Xu, X. J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T. & Langel, U. (1998). Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat Biotechnol 16(9), 857-61.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. Cold Spring Harbour Press, Cold Spring Harbour, NY.

Schwarze, S.R., Hruska, K.A. & Dowdy, S.F. Protein transduction: unrestricted delivery into all cells? Trends Cell. Biol. 10, 290-295 (2000).

Sekiguchi, M. & Iida, S. Mutants of Escherichia coli permeable to actinomycin. Proc. Natl Acad. Sci. U S A 58, 2315-2320 (1967).

Tan, Y.T., Tillett, D.J. & McKay, I.A. Molecular strategies for overcoming antibiotic resistance in bacteria. Mol. Med. Today 6, 309-314 (2000).

Taylor, R.W., Chinnery, P.F., Turnbull, D.M. & Lightowlers, R.N. Selective inhibition of mutant human mitochondrial DNA replication in vitro by peptide nucleic acids. Nature Genetics 15, 212-215 (1997).

Tegmark, K., Karlsson, A., Arvidson, S. (2000) Identification and characterization of SarH1, a new global regulator of virulence gene expression in Staphylococcus aureus. Mol. Microbiol. 37: 398-409.

Vaara, M. & Porro, M. (1996) Group of peptides that act synergistically with hydrophobic antibiotics against gram-negative enteric bacteria. Antimicrob. Agents Chemother. 40, 1801-1805.

Wagner, E. G. & Simons, R. W. (1994). Antisense RNA control in bacteria, phages, and plasmids. Annu Rev Microbiol 48, 713-42.

Wagner, R.W., Matteucci, M.D., Grant, D., Huang, T. & Froehler, B.C. Potent and selective inhibition of gene expression by an antisense heptanucleotide. Nature Biotechnol. 14, 840-844 (1996).

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. Nature 406(6797), 775-81.

White, D.G. et al. Inhibition of the multiple antibiotic resistance (mar) operon in Escherichia coli by antisense DNA analogs. Antimicrob. Agents Chemother. 41, 2699-2704 (1997).

Wittung, P., Nielsen, P.E., Buchardt, O., Egholm, M. & Norden, B. DNA-like double helix formed by peptide nucleic acid. Nature 368, 561-563 (1994).

Claims

1. A method for selecting genetically modified cells, wherein the selection is performed by
   a) modifying cells, containing a growth essential gene X, with a vector containing a gene Y, and
   b) treating said modified cells with an antisense or antigene construct directed against said essential gene X of the cells,

thereby obtaining a preferential growth of the modified cells over other, non-modified cells.

2. A method according to claim 1, wherein gene Y on the vector is able to complement gene X.

3. A method according to claims 1 or 2, wherein Y is a mutated but functionally equivalent version of gene X, herein defined as X'.

4. A method according to any one of claims 1-3, wherein the mutated gene X' on the vector is a silent mutation of gene X.

5. A method according to any of the preceding claims, wherein said antisense or antigene construct down-regulates the expression of gene X.

6. A method according to claim 5, wherein said antisense or antigene construct is selected from the group consisting of oligonucleotides, oligonucleotide analogues, oligonucleotide mimics, DNA minor groove binding polyamides etc.

7. A method according to any of claims 5 or 6, wherein said antisense or antigene construct is selected from the group consisting of PNA, LNA, phophorothioate, 2'-methoxy-, 2'-methoxyetoxy-, morpholino, phophoramidate etc.

8. A method according to any of claims 5 to 7, wherein said antisense construct is a PNA.

9. A method according to any of claims 5 to 7, wherein said antisense construct is a modified PNA.

10. A method according to claim 9, wherein the modification is obtained by linking the PNA to a peptide, a carbonhydrate or a lipid.

11. A method according to claims 9, wherein the modification is obtained by internal modification i.e. backbone modification, in which the glycine of the PNA backbone is exchanged for other α-amino acids.

12. A method according to any of the preceding claims, wherein said antisense construct consists of 5-30 nucleobase units.

13. A method according to any of the preceding claims, wherein said antisense construct consists of 8-15 nucleobase units.

14. A method according to any of the preceding claims, wherein said antisense construct consists of 10-12 nucleobase units.

15. A method according to any of claims 8-14, wherein said antisense construct is a PNA covalently linked to a cell wall or cell membrane transport peptide.

16. A method according to claim 15, wherein said antisense construct is a PNA covalently linked to a signal peptide comprising the aminoacid sequence: KFFKFFKFFK (SEQ ID NO: 1) or KFFKFFKFFKC (SEQ ID NO: 2) or IKFLKFLKFLC (SEQ ID NO: 3) or VDKGSYLPRPTPPRPIYNC (SEQ ID NO: 4) or ILPWKWPWWPWRRGC (SEQ ID NO: 5) or KLAKALKKLLC (SEQ ID NO: 6).

17. A method according to claim 15, wherein said antisense construct is a PNA covalently linked to a cell wall or cell membrane transport peptide comprising the aminoacid sequence: KFFKFFKFFK (SEQ ID NO: 1).

18. A method according to any of the preceding claims, wherein said antisense construct is directed to the site of the start codon of said essential gene X.

19. A method according to any of the preceding claims, wherein said antisense construct is directed to the site of the startcodon of said essential gene X and result in inhibition of the translation of said essential gene X when hybridised to gene X.

20. A method according to any of the preceding claims, wherein said essential gene X is selected from the goup of genes consisting of essential genes of : *Escherichia* species and subspecies including *E. coli* and *E. coli* K12, *Bacillus* species and subspecies including *B. subtilis*, *Saccharomyces* species including *S. cerevisae* and *S. carlsbergensis*, *Schizosaccharomyces* species including *Schizosaccharomyces pombe*, *Lactococcus* species including *Lactococcus lactis* and *Lactococcus lactis* subsp. *cremoris*, *Streptococcus* species,

Enterococcus species, *Lactobacillus* species, *Leuconostoc* species, *Oenococcus* species, *Pediococcus* species, *Bifidobacterium* species, *Pseudomonas* species including *Pseudomonas aeruginosa* and *Pseudomonas acidovorans*, *Chromobacterium* species such as *Chromobacterium violaceans*, *Kluyyeromyces* species including *K. lactis*, *Pichia* species including *Pichia pastoris*, *Hansenula* species including *Hansenula polymorpha*, *Yarowia, Schwaniomyces* and *Zygosaccharomyces* species.

21. A method according to any of claims 1-19 , wherein said essential gene X is selected from the group of genes consisting of the *E. coli* genes: *acpP*, *accD, acpS, frr, infA, lnt, murA, murI, parC, proS, rpoE* and *rpsB.*

22. A method according to any of claims 1-19 , wherein said essential gene X is the *Bacillus subtilis* genes *acpA, sigH, nadE, sbp, ftsZ, sipS, sigA, tagH* and *tagG.*

23. A method according to any of claims 1-19 , wherein said essential gene X is selected from the group of genes consisting of: *Saccharomyces cerevisae* gene *ACC1*, *TOP2, ALA1, CDC4, CDC28, RPC25, PMI40, PAP1, TFC8, YKT6, TAF145* and *TIF11*.

24. A method according to any of the preceding claims, wherein said antisense construct selected from the group of constructs having the nucleobase sequence: (5'-/N-)CTCATACTCT(3'-/N-) (SEQ ID NO: 7), (5'-/N-)CATACTCTTAAA(3'-/C-) (SEQ ID NO: 8), (5'-/N-)CCTATCAAAACT(3'-/C-) (SEQ ID NO: 9), (5'-/N-)TGCTCATACTCT(3'-/C-) (SEQ ID NO: 10), (5'-/N-)ACTCTTAAATTT(3'-/C-) (SEQ ID NO: 11), (5'-/N-)AGTGCTCATACT(3'-/C-) (SEQ ID NO: 12), (5'-/N-)TCTTCGATAGTG(3'-/C-) (SEQ ID NO: 13) or (5'-/N-)GATAGTGCTCAT(3'-/C-) (SEQ ID NO: 14).

25. A method according to any of claims 1-23, wherein the antisense construct is selected from the group of constructs having the nucleobase sequence: (5'-/N-) CTCATACTCT(3'-/N-) (SEQ ID NO: 7), (5'-/N-)CATACTCTTAAA(3'-/C-) (SEQ ID NO: 8) or (5'-/N-) CCTATCAAAACT(3'-/C-) (SEQ ID NO: 9).

26. A method according to any of claims 1-23, wherein said antisense construct comprises the nucleobase sequence: (5'-/N-) CTCATACTCT (3'-/C-) (SEQ ID NO: 7),.

27. A method according to any of the preceding claims, wherein gene X' is a mutated version of gene X, coding for the wild-type product of gene X but mutated at one or more positions between the Shine-Delgarno sequence and the start codon of the gene.

28. A method according to any of the preceding claims, wherein gene X' comprises the sequence 5'-AACAGAATTCATGAGCACTA-3' (SEQ ID NO: 19).

29. A method according to any of claims 1-5, wherein said antisense construct is selected from the group of constructs consisting of: PNA#1934 H-KFFKFFKFFK-eg1-jtjtjjt-$(eg1)_3$-tcctctc- lysNH_2 (SEQ ID NO: 21) and PNA#SP4 H-KFFKFFKFFK-eg1-ctcatactct-NH_2 (SEQ ID NO: 20) and wherein j bases indicate pseudo-isocytosine and eg1 is a flexible ethylene glycol-lysine linker.

30. A product manufactured fully or partially by use of a method according to any of the preceding claims

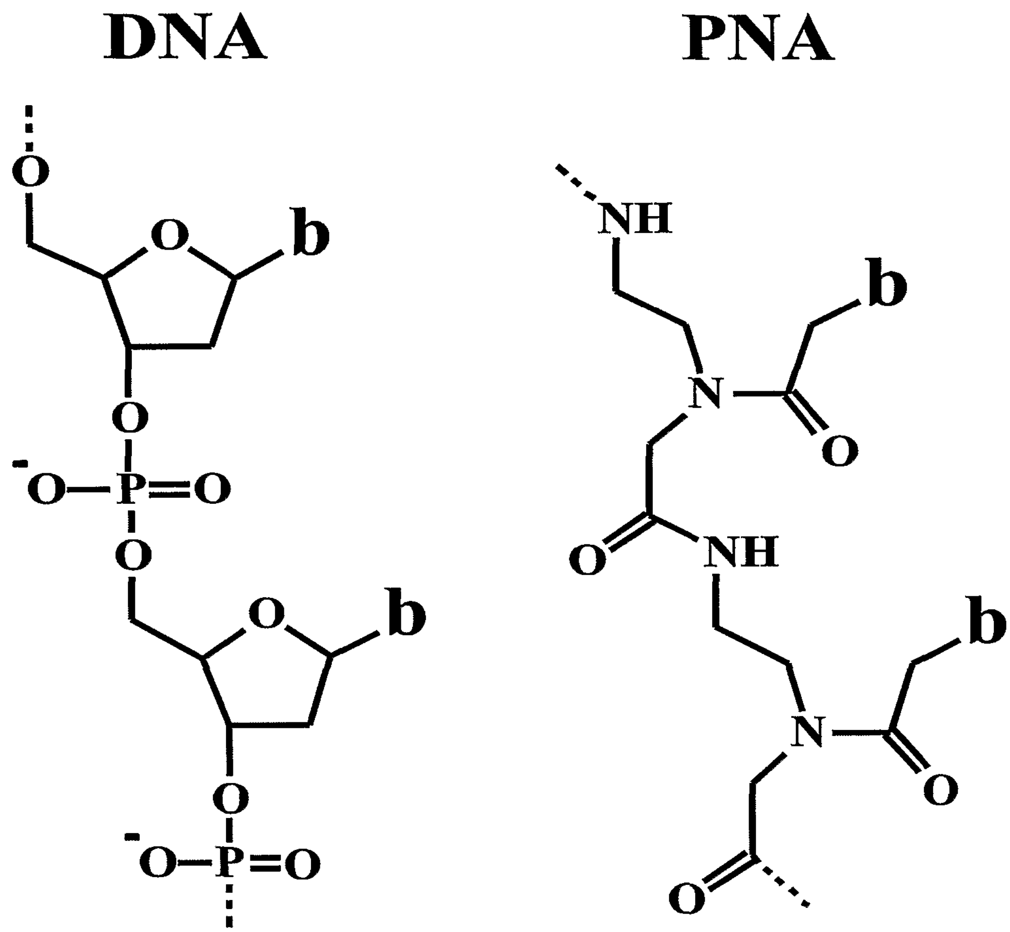


Fig. 1

Wild type *E. coli* K12

chromosomal *acpP* mRNA

```
5' ..AUUUAAGAGUAUGAGCACUA....
         ||||||||||
         TCTCATACTC-o-(KFF)3K-(N)
         peptide-PNA #SP4
```

E. coli K12 carrying *acpP-1*

chromosomal *acpP* mRNA

```
5' ..AUUUAAGAGUAUGAGCACUA....
         ||||||||||
         TCTCATACTC-o-(KFF)3K-(N)
         peptide-PNA #SP4
```

plasmid-bourne *acpP-1* mRNA

```
5' ..AACAGAAUUCAUGAGCACUA....
              |||||
         TCTCATACTC-o-(KFF)3K-(N)
         peptide-PNA #SP4
```

Fig. 2

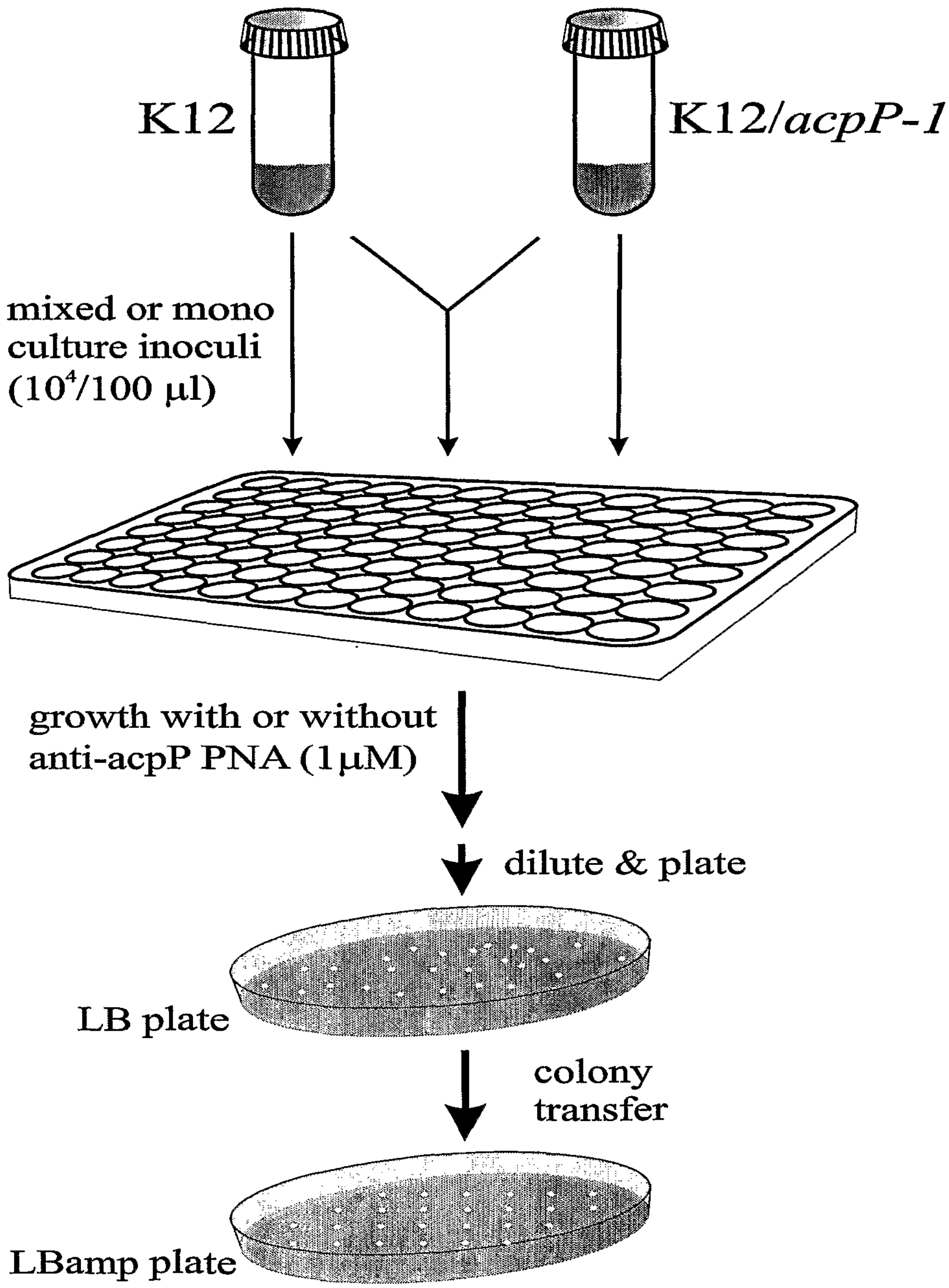

K12
K12/acpP-1
mixed or mono culture inoculi (10^4/100 µl)
growth with or without anti-acpP PNA (1µM)
dilute & plate
LB plate
colony transfer
LBamp plate

Fig. 3

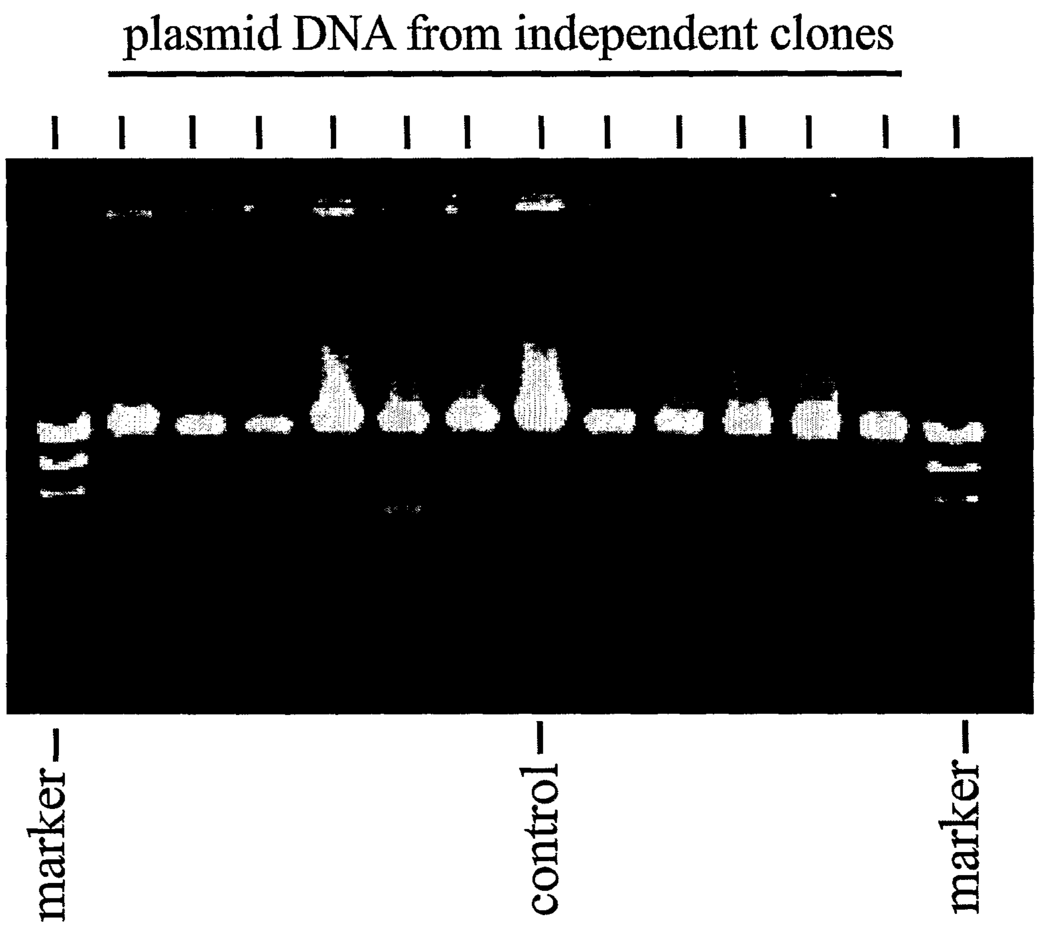

plasmid DNA from independent clones
marker
control
marker

Fig. 4

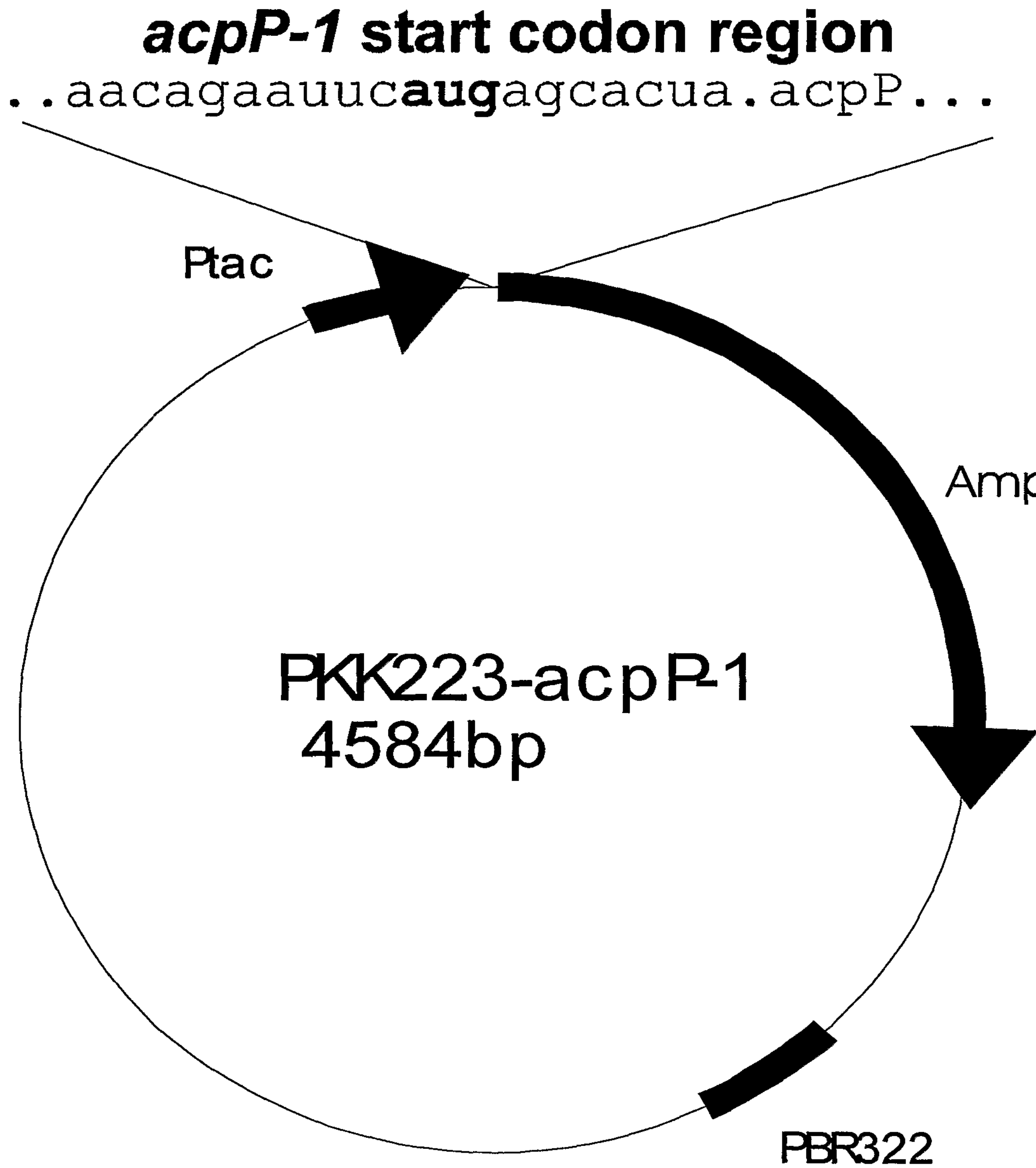
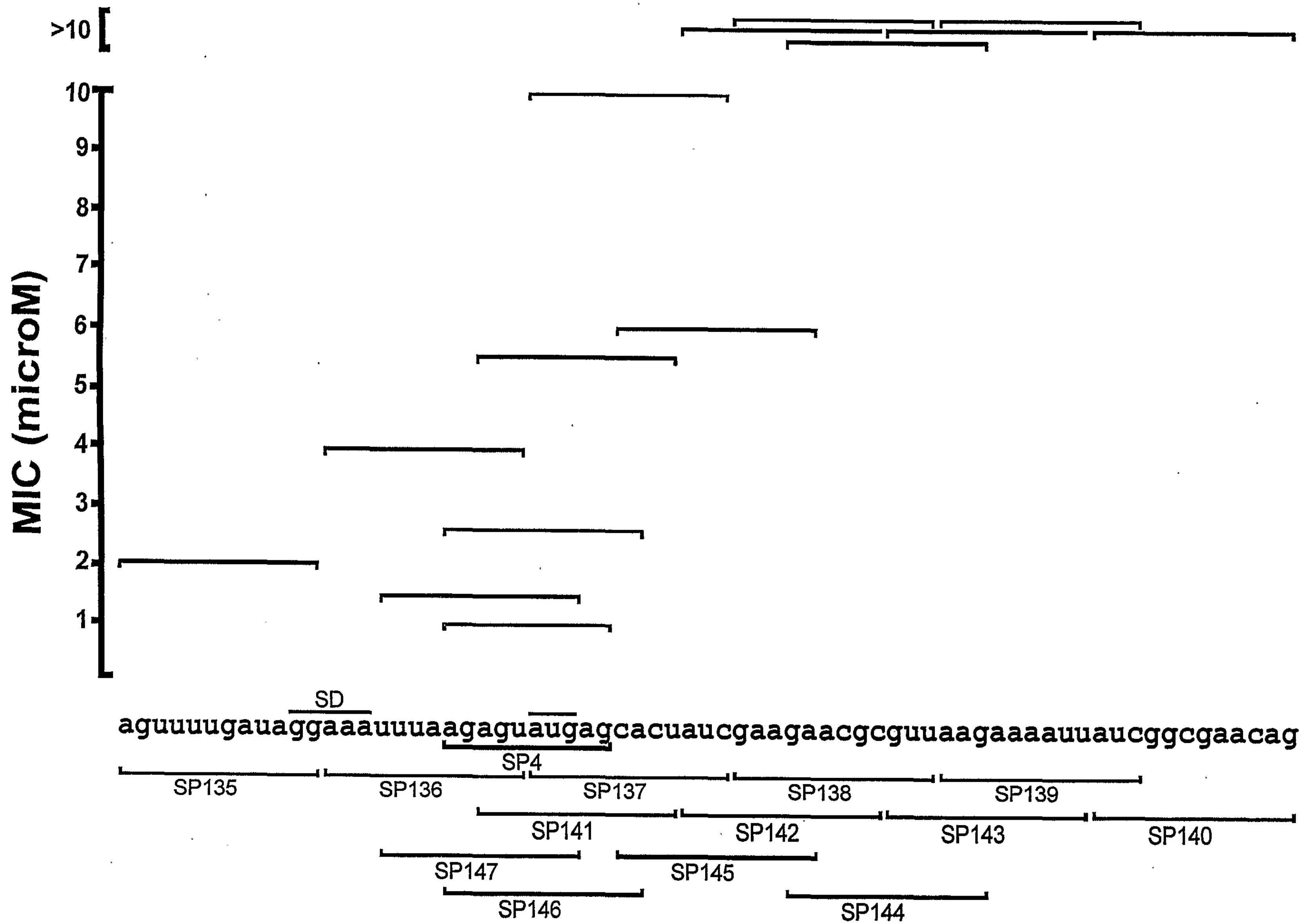

acpP-1 start codon region
..aacagaauuc**aug**agcacua.acpP...
Ptac
Ampr
PKK223-acpP-1
4584bp
PBR322

Fig. 5



>10
10
9
8
7
6
5
4
3
2
1
MIC (microM)
SD
aguuuugauaggaaauuuaagaguaugagcacuaucgaagaacgcguuaagaaaauuaucggcgaacag
SP4
SP135
SP136
SP137
SP138
SP139
SP141
SP142
SP143
SP140
SP147
SP145
SP146
SP144

Fig. 6

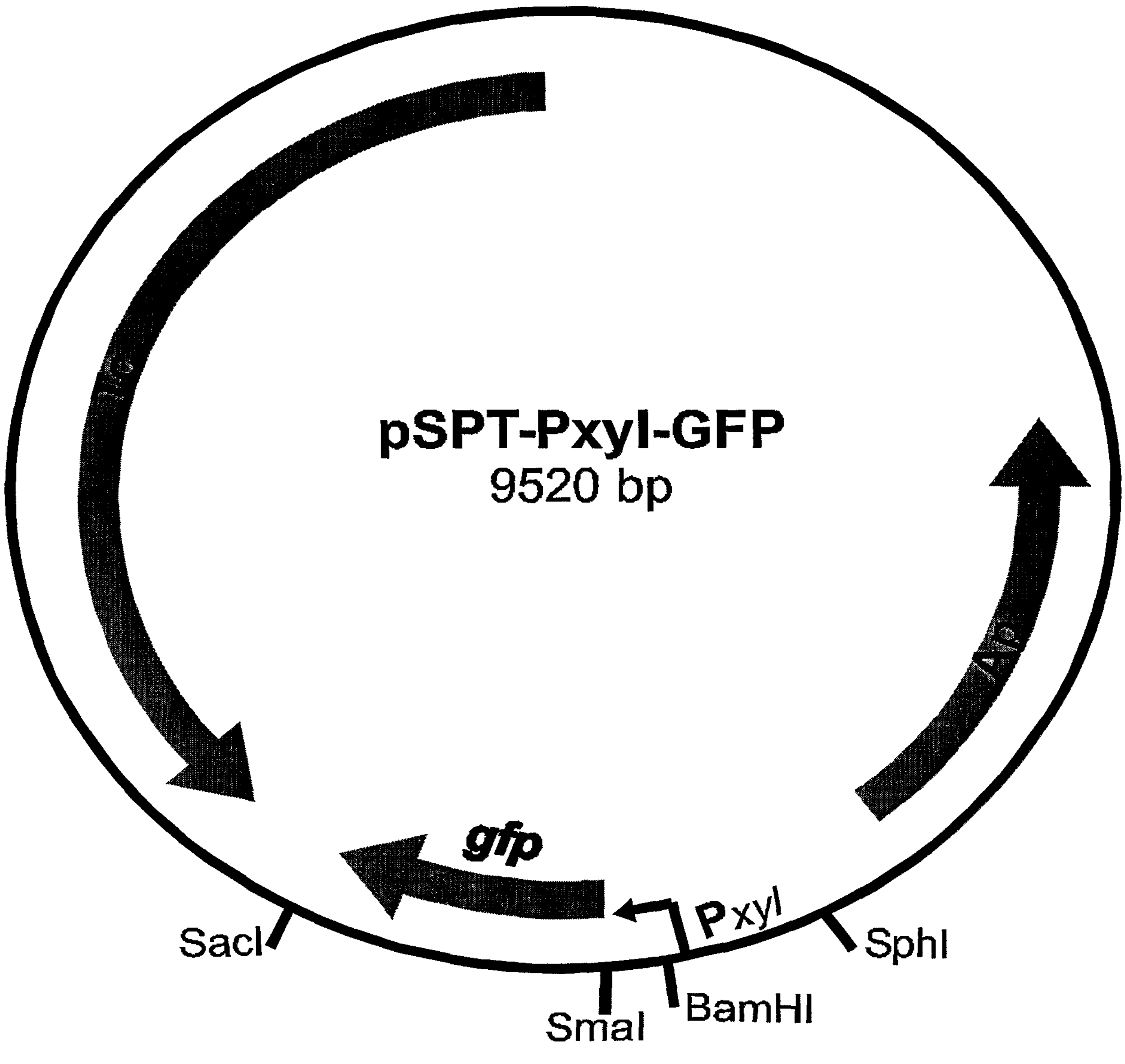


Fig. 7

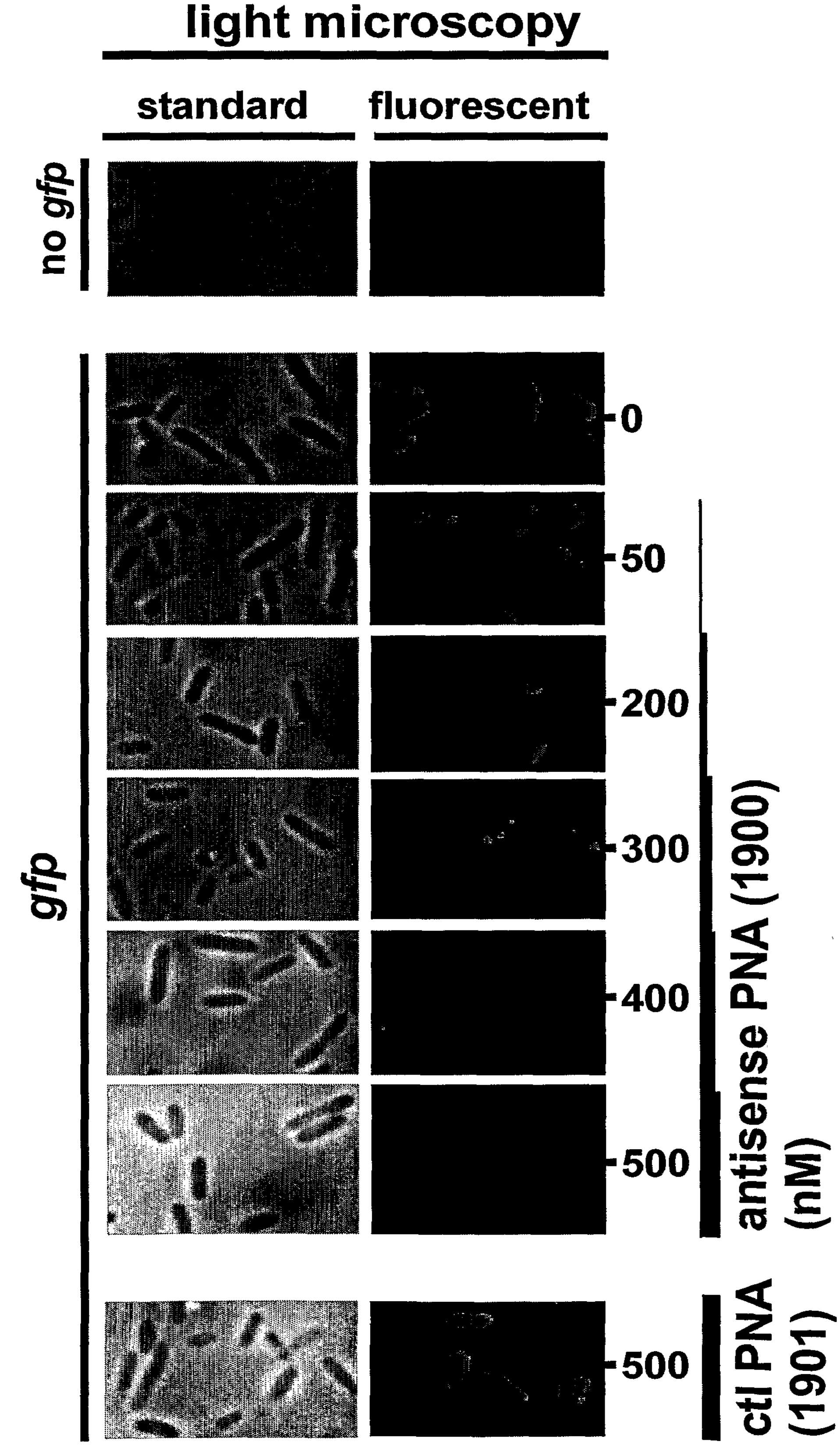

light microscopy
standard
fluorescent
no gfp
gfp
0
50
200
300
400
500
500
antisense PNA (1900) (nM)
ctl PNA (1901)

Fig. 8

5'. gaaacagcuaugaccau........	*lacZ* mRNA
ctttgtcgatactgg-(N)	15 mer anti-*lacZ* PNA 1284
ctttgtcgatac-(N)	12 mer anti-*lacZ* PNA 1834
ctttgtcgatac-eg1-KFFKFFKFFK-(N)	12 mer anti-*lacZ* peptide-PNA 1900

Fig. 9

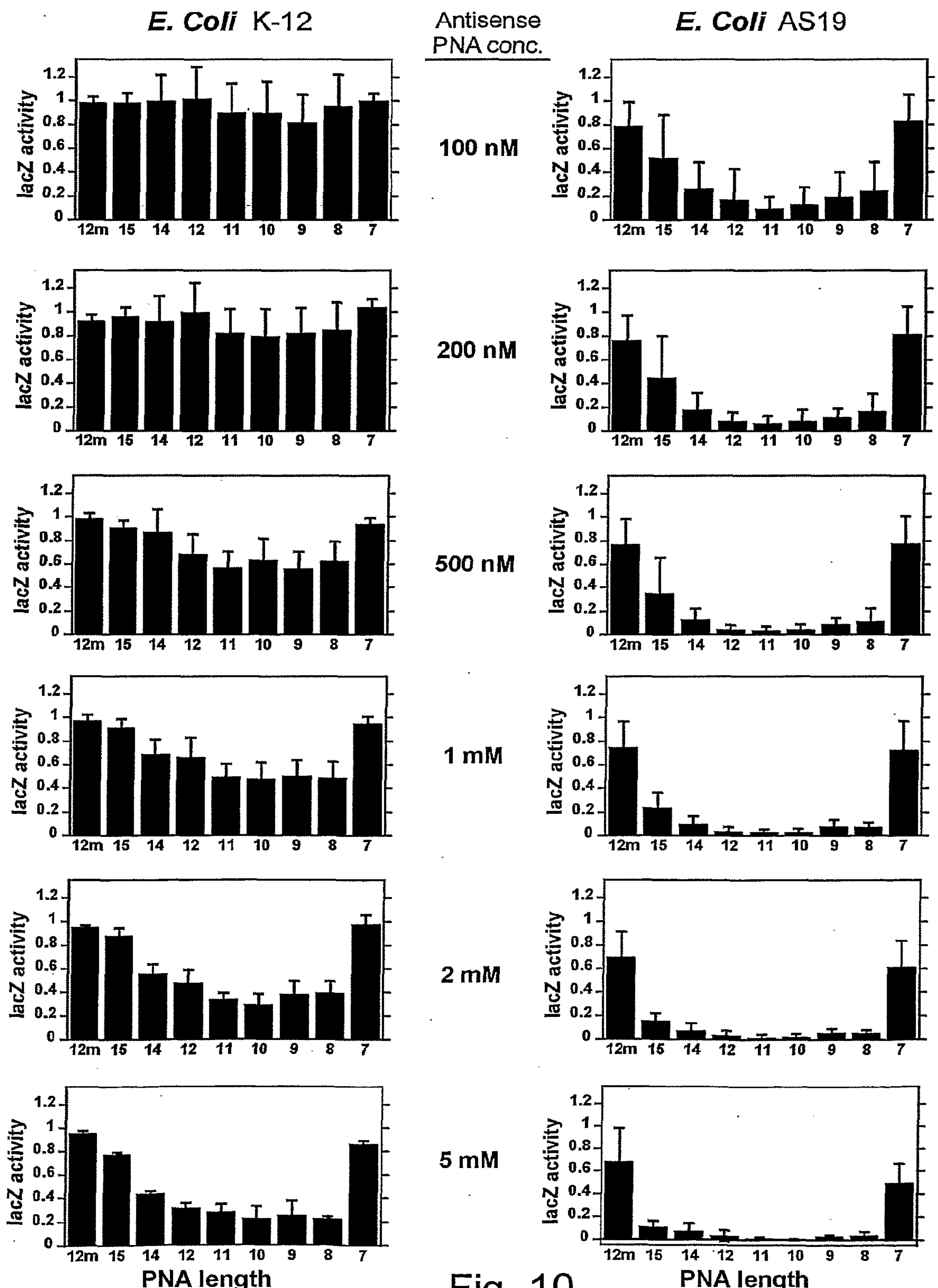

E. Coli K-12
Antisense PNA conc.
E. Coli AS19
100 nM
200 nM
500 nM
1 mM
2 mM
5 mM
lacZ activity
12m 15 14 12 11 10 9 8 7
PNA length

Fig. 10

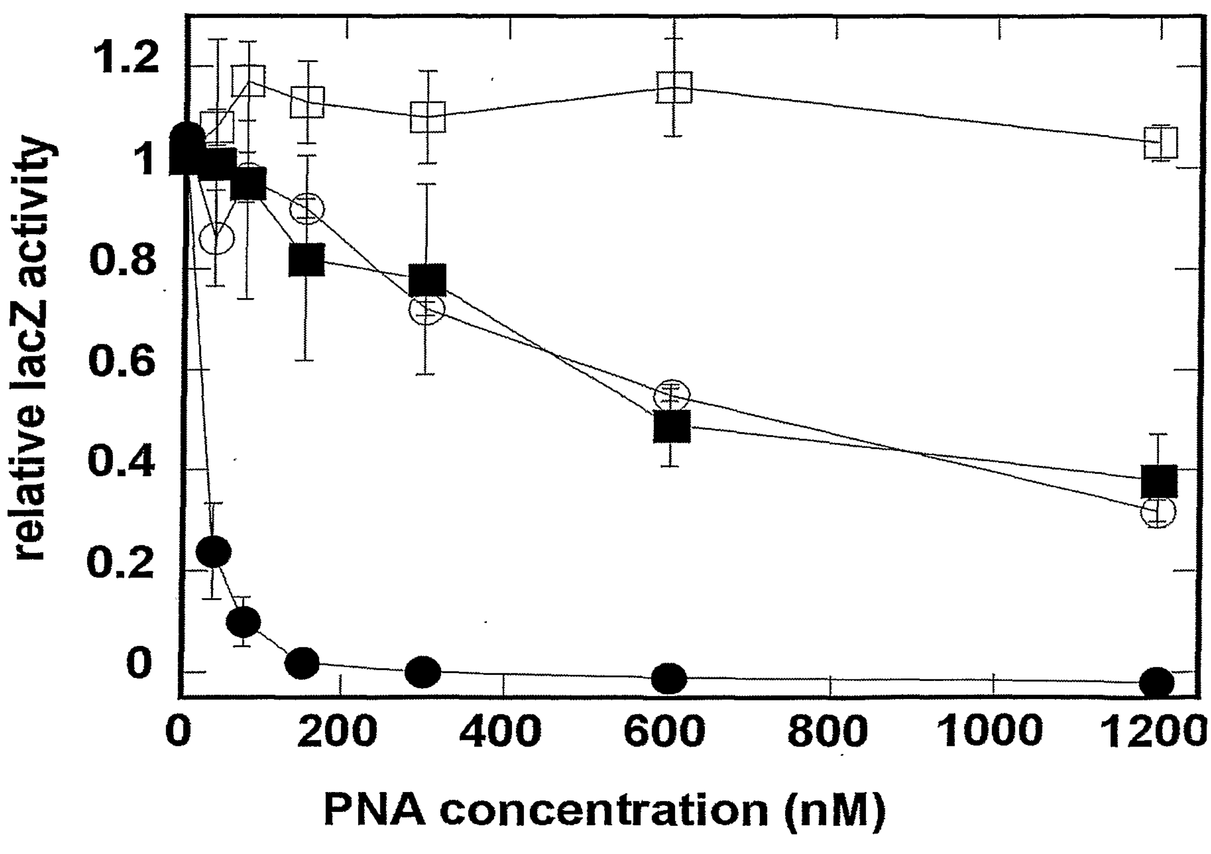

1.2
1
0.8
0.6
0.4
0.2
0
relative lacZ activity
0
200
400
600
800
1000
1200
PNA concentration (nM)

Fig. 11

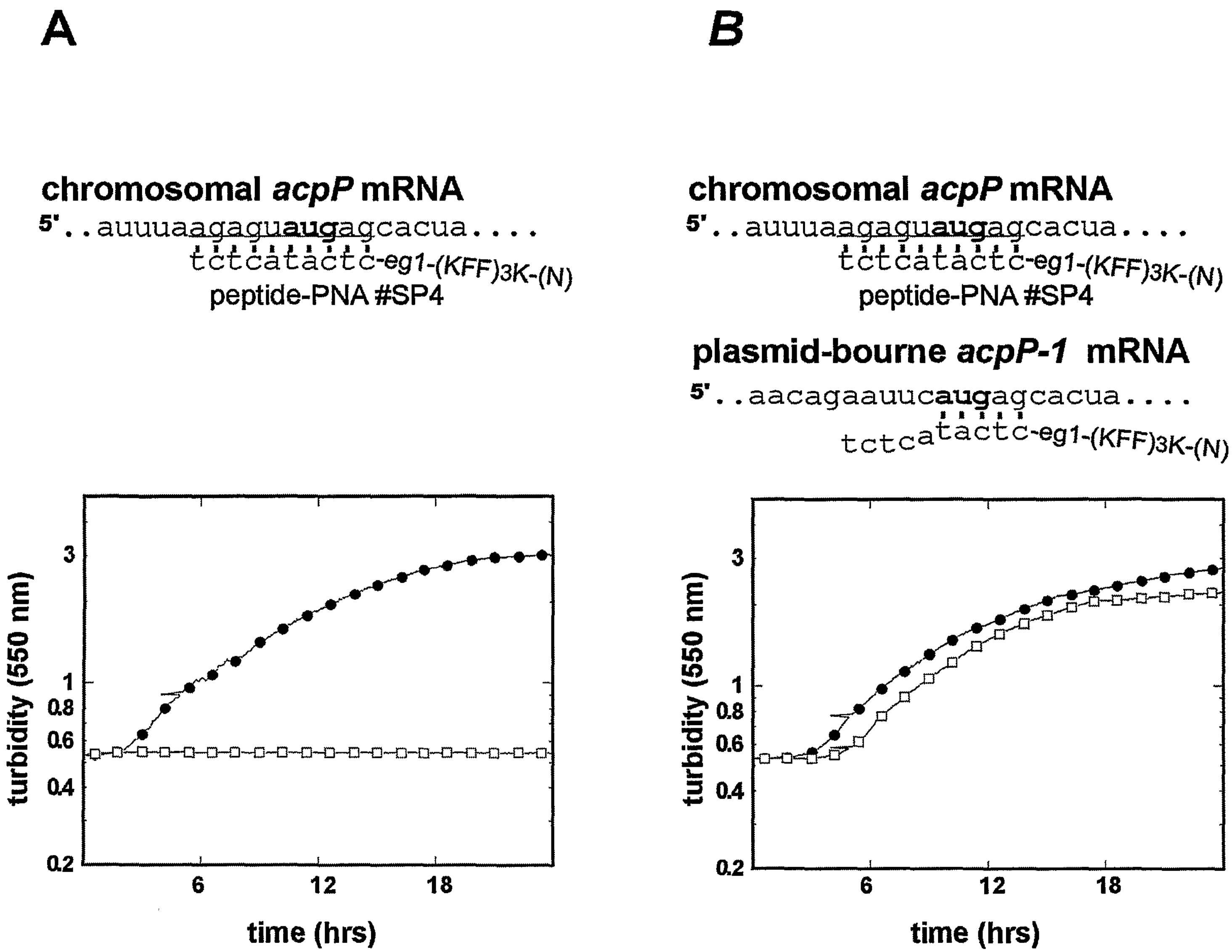

A
chromosomal acpP mRNA
5'..auuuaagaguaugagcacua....
tctcatactc-eg1-(KFF)3K-(N)
peptide-PNA #SP4
turbidity (550 nm)
3
1
0.8
0.6
0.4
0.2
6
12
18
time (hrs)
B
chromosomal acpP mRNA
5'..auuuaagaguaugagcacua....
tctcatactc-eg1-(KFF)3K-(N)
peptide-PNA #SP4
plasmid-bourne acpP-1 mRNA
5'..aacagaauucaugagcacua....
tctcatactc-eg1-(KFF)3K-(N)
turbidity (550 nm)
3
1
0.8
0.6
0.4
0.2
6
12
18
time (hrs)

Fig. 12

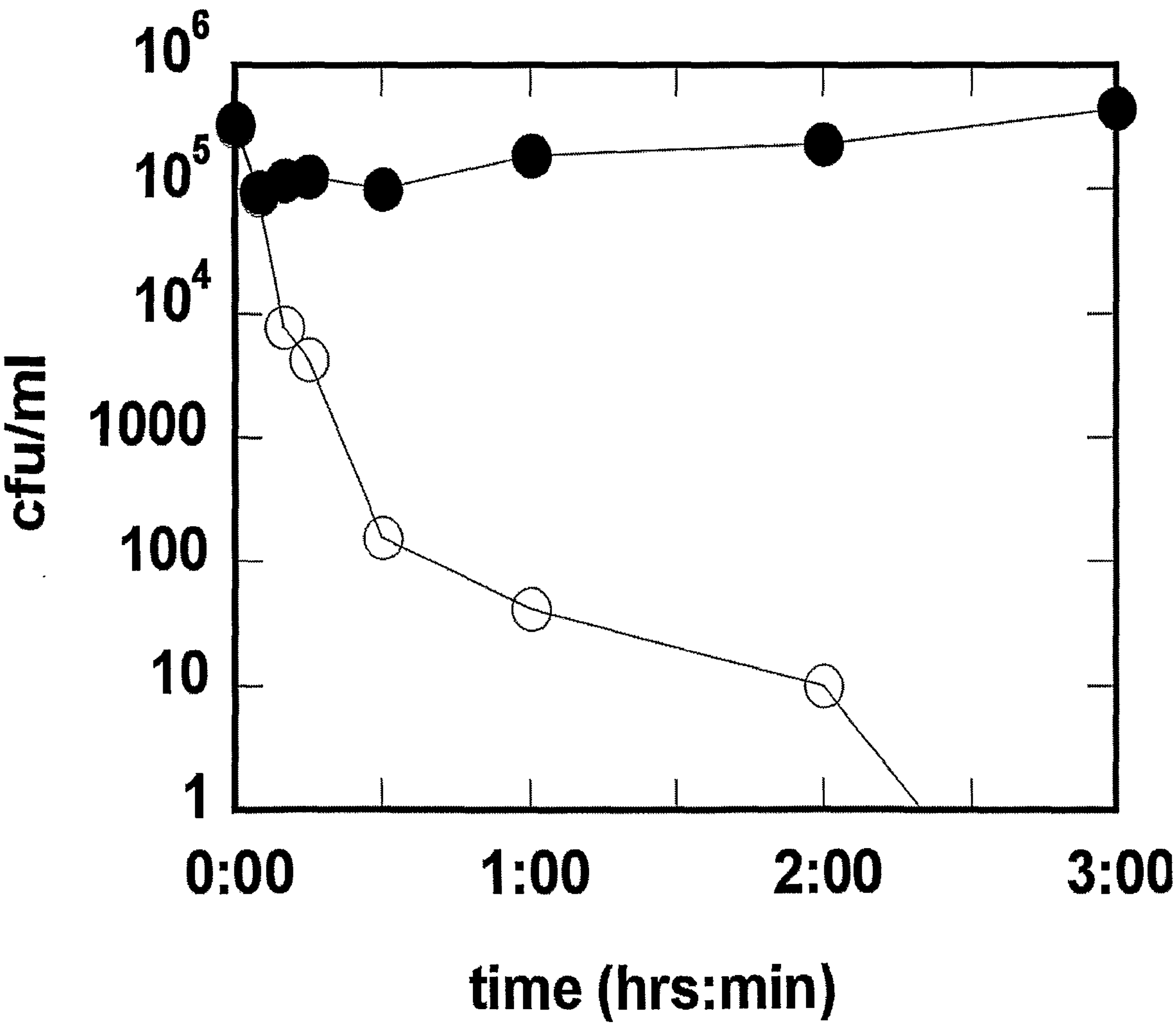


Fig. 13

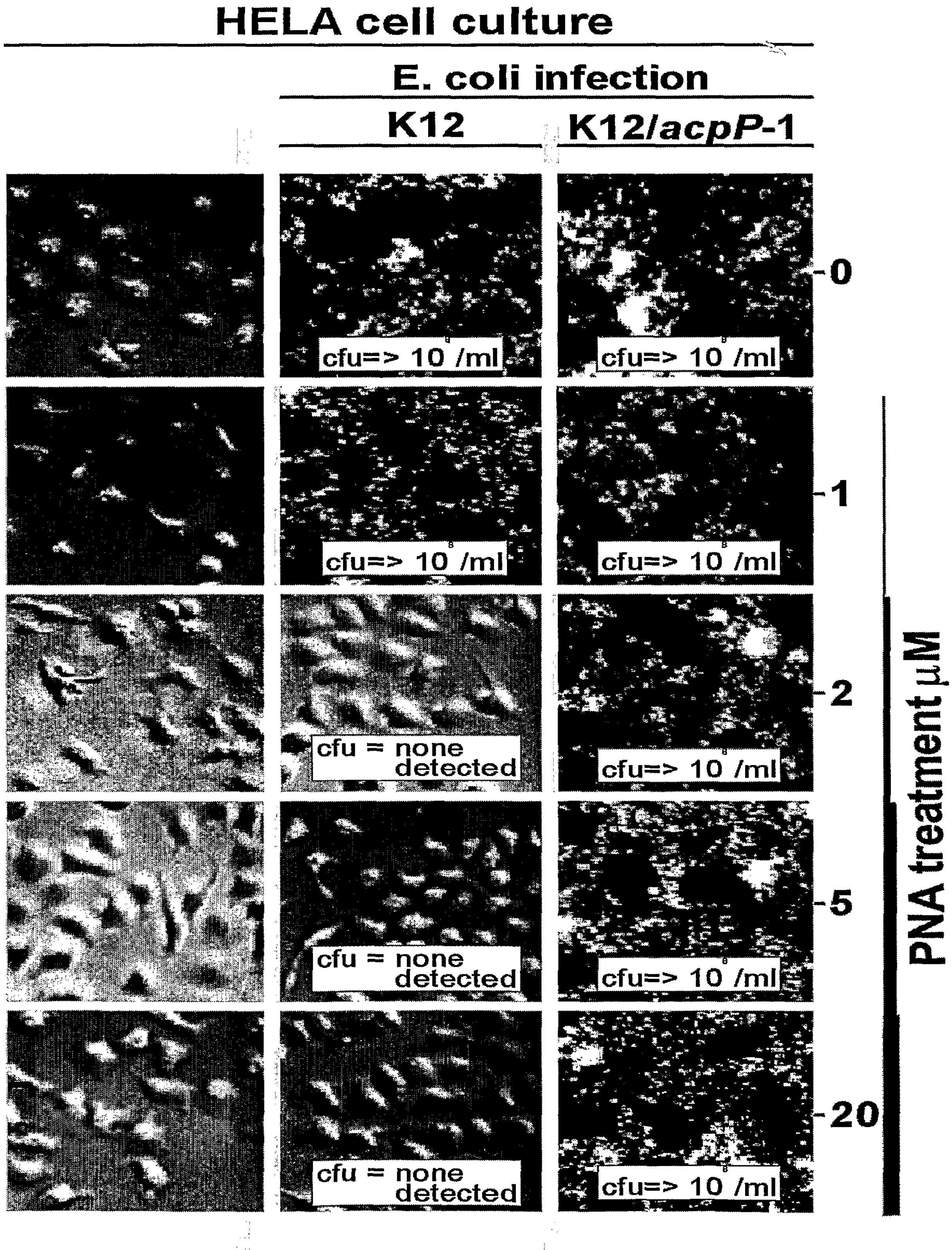
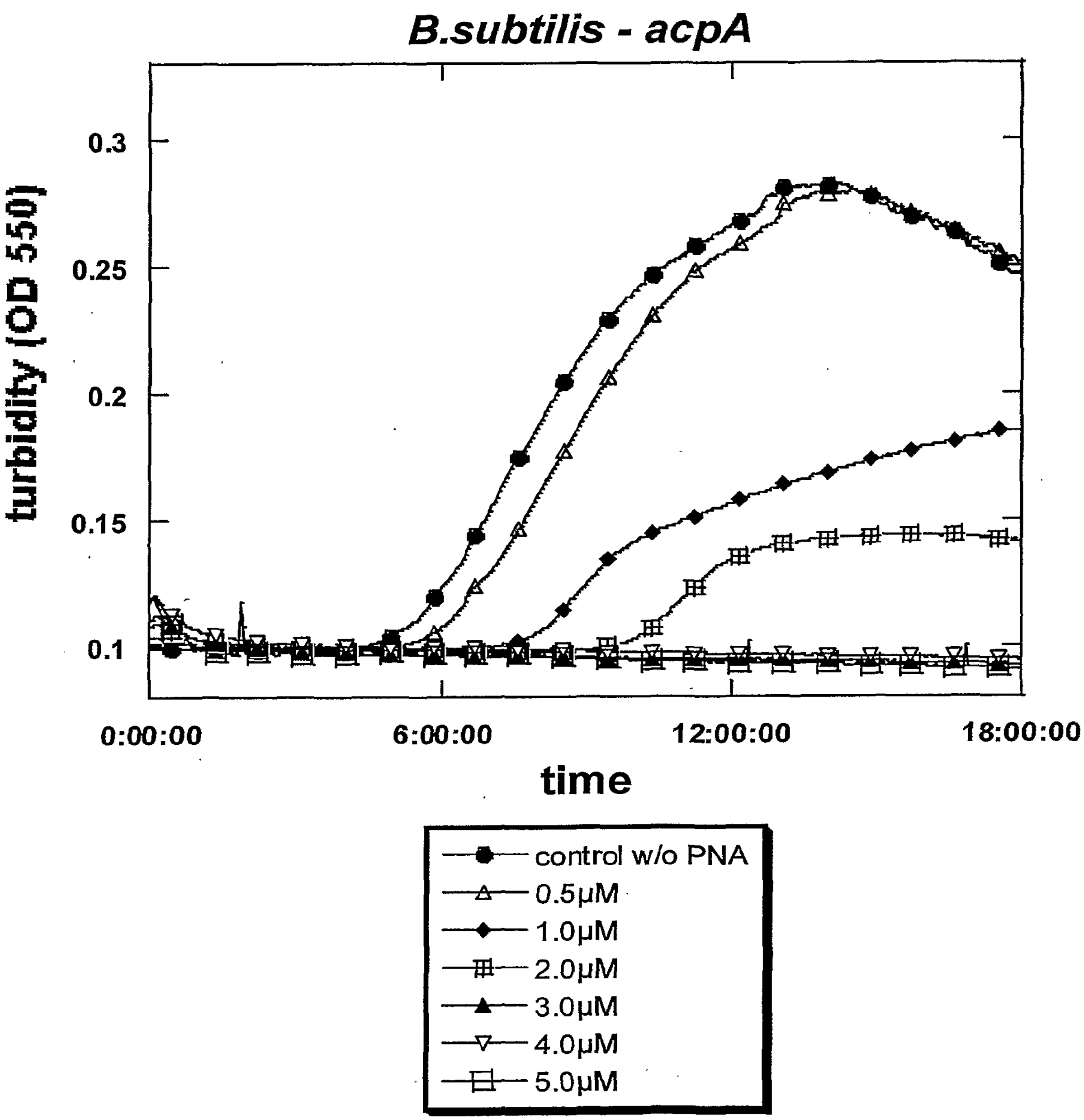

HELA cell culture
E. coli infection
K12
K12/acpP-1
cfu=> 10^8 /ml
cfu=> 10^8 /ml
cfu=> 10^8 /ml
cfu=> 10^8 /ml
cfu = none detected
cfu=> 10^8 /ml
cfu = none detected
cfu=> 10^8 /ml
cfu = none detected
cfu=> 10^8 /ml
0
1
2
5
20
PNA treatment μM

Fig. 14



B.subtilis - acpA
turbidity (OD 550)
0.3
0.25
0.2
0.15
0.1
0:00:00
6:00:00
12:00:00
18:00:00
time
control w/o PNA
0.5µM
1.0µM
2.0µM
3.0µM
4.0µM
5.0µM

Fig. 15

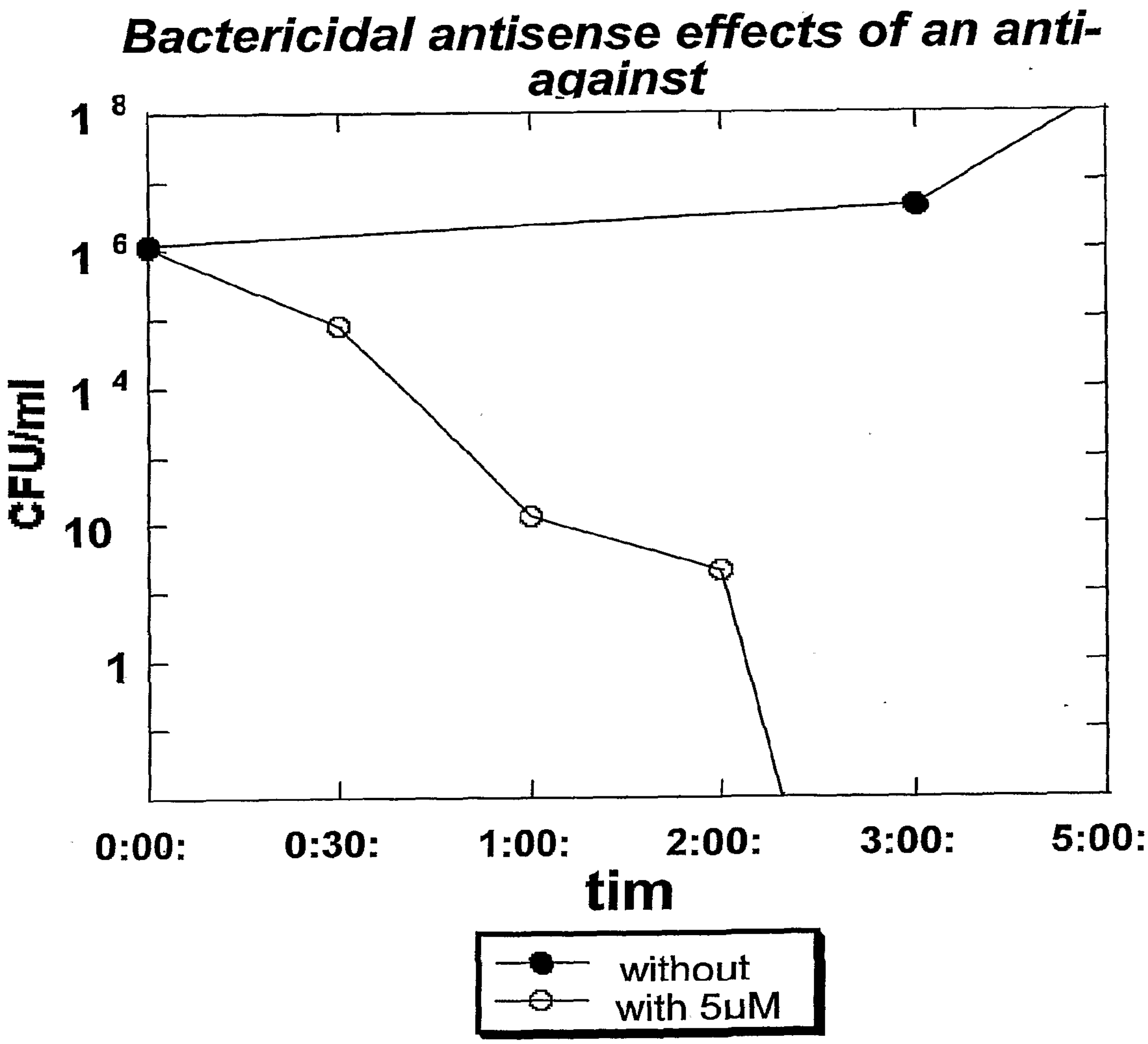

Bactericidal antisense effects of an anti-
against
CFU/ml
1 8
1 6
1 4
10
1
0:00:
0:30:
1:00:
2:00:
3:00:
5:00:
tim
without
with 5µM

Fig. 16

SEQUENCE LISTING

<110> Nielsen, Peter E.
Good, Liam

<120> Antibiotic-free bacterial strain selection with antisense molecules

<130> 25893PC01

<150> PA 2001 00523
<151> 2001-03-29

<160> 52

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> cell membrane transport peptide

<400> 1
Lys Phe Phe Lys Phe Phe Lys Phe Phe Lys
1 5 10

<210> 2
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic cell membrane transport peptide

<400> 2

Lys Phe Phe Lys Phe Phe Lys Phe Phe Lys Cys
 1 5 10

<210> 3
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic cell membrane transport peptide

<400> 3

Ile Lys Phe Leu Lys Phe Leu Lys Phe Leu Cys
 1 5 10

<210> 4
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic cell membrane transport peptide

<400> 4

Val Asp Lys Gly Ser Tyr Leu Pro Arg Pro Thr Pro Pro Arg Pro Ile
 1 5 10 15
Tyr Asn Cys

<210> 5
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic cell membrane transport peptide

<400> 5
Ile Leu Pro Trp Lys Trp Pro Trp Trp Pro Trp Arg Arg Gly Cys
1 5 10 15

<210> 6
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic cell membrane transport peptide

<400> 6
Lys Leu Ala Lys Ala Leu Lys Lys Leu Leu Cys
1 5 10

<210> 7
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 7
ctcatactct 10

<210> 8
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 8
catactctta aa 12

<210> 9
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 9
cctatcaaaa ct 12

<210> 10
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 10
tgctcatact ct 12

<210> 11
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 11
actcttaaat tt 12

<210> 12
<211> 12

<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 12
agtgctcata ct 12

<210> 13
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 13
tcttcgatag tg 12

<210> 14
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic antisense oligonucleotide

<400> 14
gatagtgctc at 12

<210> 15
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide acpP primer

<400> 15
gagaattcat gagcactatc gaagaac 27

<210> 16
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide acpP primer

<400> 16
agttaagctt gaccgcctgg agatgtt 27

<210> 17
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide, PCR primer for cloning

<400> 17
aaactgcagg aggaaacagc tatgctcgag atatcgaagg gcgaggag 48

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide, PCR primer for cloning

<400> 18
gatgagctct cggctcgatg atcc 24

<210> 19
<211> 20
<212> DNA

<213> Artificial Sequence

<220>
<223> plasmid-bourne mutated acpP sequence (acpP-1)

<400> 19
aacagaattc atgagcacta 20

<210> 20
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid SP4

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker called "eg1"

<400> 20
ctcatactct 10

<210> 21
<211> 7
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid 1934

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via a eg1-jtjtjjt-(eg1)(eg1)(eg1) linker. Wherein "j"

indicate pseudo-isocytosine PNA and "t" a thymine PNA, "eg1" indicate a ethylene glycol linker.

<400> 21
tcctctc 7

<210> 22
<211> 20
<212> RNA
<213> Escherichia coli

<220>
<221> misc_feature
<222> (0)...(0)
<223> Part of AcpP mRNA

<400> 22
auuuaagagu augagcacua 20

<210> 23
<211> 20
<212> RNA
<213> Artificial Sequence

<220>
<223> part of plasmid encoded acpP-1 mRNA

<400> 23
aacagaauuc augagcacua 20

<210> 24
<211> 17
<212> RNA
<213> Escherichia coli

<220>
<221> misc_feature
<222> (0)...(0)
<223> part of LacZ mRNA

<400> 24
gaaacagcua ugaccau 17

<210> 25
<211> 69
<212> RNA
<213> Esherichia coli

<220>
<221> misc_feature
<222> (0)...(0)
<223> E. coli acpP mRNA 5' region

<400> 25
aguuuugaua ggaaauuuaa gaguaugagc acuaucgaag aacgcguuaa gaaaauuauc 60
ggcgaacag 69

<210> 26
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid 1432

<221> misc_feature
<222> 15
<223> A lysine residue is linked to COOH-terminal of the
PNA sequence

<400> 26
ggtcatagct gtttc 15

<210> 27
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1835

<221> misc_feature
<222> 14
<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 27
gtcatagctg tttc 14

<210> 28
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1834

<221> misc_feature
<222> 12
<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 28
catagctgtt tc 12

<210> 29
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1877

<221> misc_feature
<222> 11
<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 29
atagctgttt c 11

<210> 30
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1833

<221> misc_feature
<222> 10
<223> A lysine residue is linked to COOH-terminal of the PNA

<400> 30
tagctgtttc 10

<210> 31
<211> 9
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1876

<221> misc_feature
<222> 9
<223> A lysine residue is linked to COOH-terminal of the PNA

<400> 31
agctgtttc 9

<210> 32
<211> 8
<212> DNA

<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1832

<221> misc_feature
<222> 8
<223> A lysine residue is linked to COOH-terminal of the PNA

<400> 32
gctgtttc 8

<210> 33
<211> 7
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1875

<221> misc_feature
<222> 7
<223> A lysine residue is linked to COOH-terminal of the PNA

<400> 33
ctgtttc 7

<210> 34
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1878

<221> misc_feature
<222> 12

<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 34
caatgtcgtt tc 12

<210> 35
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 111

<221> misc_feature
<222> 12
<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 35
cccctattgt cc 12

<210> 36
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1176

<221> misc_feature
<222> 15
<223> A lysine residue is linked to COOH-terminal of the
PNA

<400> 36
gcaagcgact gtgga 15

<210> 37

<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1873

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 37
ggtcatagct gtttc 15

<210> 38
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. SP183

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 38
gtgactcgat gtctt 15

<210> 39
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Peptide nucleic acid no. 1900

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 39
catagctgtt tc 12

<210> 40
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1901

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 40
caatgtcgtt tc 12

<210> 41
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. SP182

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked

to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 41
acatgtcgtc tt 12

<210> 42
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1978

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFKC (SEQ ID NO:2) is linked to N-terminal of the PNA sequence via a maleimide SMCC coupling.

<400> 42
catagctgtt tc 12

<210> 43
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. p29

<221> misc_feature
<222> 1
<223> The polypeptide IKFLKFLKFLC (SEQ ID NO:3) is linked to N-terminal of the PNA sequence via a maleimide SMCC coupling.

<400> 43
catagctgtt tc 12

<210> 44
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. p30

<221> misc_feature
<222> 1
<223> The polypeptide VDKGSYLPRPTPPRPIYNC (SEQ ID NO:4) is linked to N-terminal of the PNA sequence via a maleimide SMCC coupling.

<400> 44
catagctgtt tc 12

<210> 45
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. p31

<221> misc_feature
<222> 1
<223> The polypeptide ILPWKWPWWPWRRGC (SEQ ID NO:5) is linked to N-terminal of the PNA sequence via a maleimide SMCC coupling.

<400> 45
catagctgtt tc 12

<210> 46
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. p32

<221> misc_feature
<222> 1
<223> The polypeptide KLAKALKKLLC (SEQ ID NO:6) is
linked to N-terminal of the PNA sequence via a
maleimide SMCC coupling.

<400> 46
catagctgtt tc 12

<210> 47
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1143

<221> misc_feature
<222> 15
<223> A lysine residue is linked to the PNA

<221> modified_base
<222> 1
<223> n = pseudo-isocytosine

<221> modified_base
<222> 3
<223> n = pseudo-isocytosine

<221> modified_base
<222> 5
<223> n = pseudo-isocytosine

<221> modified_base
<222> 6

<223> n = pseudo-isocytosine

<221> misc_feature
<222> (8)...(8)
<223> (eg1)(eg1)(eg1) linker. Wherein "eg1" indicate one
ethylene glycol linker.

<400> 47
ntntnntntc ctctc 15

<210> 48
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1410

<221> modified_base
<222> 2
<223> n = pseudo-isocytosine PNA

<221> modified_base
<222> 4
<223> n = pseudo-isocytosine PNA

<221> modified_base
<222> 5
<223> n = pseudo-isocytosine PNA

<221> misc_feature
<222> 14
<223> A lysine residue is linked to the PNA

<221> misc_feature
<222> 7
<223> (eg1)(eg1)(eg1) linker. Wherein "eg1" indicate one
ethylene glycol linker.

<400> 48
tntnntntcc tctc 14

<210> 49
<211> 13
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1406

<221> modified_base
<222> 1
<223> n = pseudo-isocytosine PNA

<221> modified_base
<222> 3
<223> n = pseudo-isocytosine PNA

<221> modified_base
<222> 4
<223> n = pseudo-isocytosine PNA

<221> misc_feature
<222> 6
<223> (eg1)(eg1)(eg1) linker. Wherein "eg1" indicate one ethylene glycol linker.

<221> misc_feature
<222> 13
<223> A lysine residue is linked to the PNA

<400> 49
ntnntntcct ctc 13

<210> 50
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. 1407

<221> modified_base
<222> 2
<223> n = pseudo-isocytosine PNA

<221> modified_base
<222> 3
<223> n = pseudo-isocytosine PNA

<221> misc_feature
<222> 5
<223> (eg1)(eg1)(eg1) linker. Wherein "eg1" indicate one
ethylene glycol linker.

<221> misc_feature
<222> 12
<223> A lysine residue is linked to the PNA.

<400> 50
tnntntcctc tc 12

<210> 51
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Peptide nucleic acid no. SP181

<221> misc_feature
<222> 1
<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked
to N-terminal of the PNA sequence via the ethylene
glycol linker "eg1".

<400> 51

tcactatctc 10

<210> 52

<211> 10

<212> DNA

<213> Artificial Sequence

<220>

<223> Peptide nucleic acid no. 2316

<221> misc_feature

<222> 1

<223> The polypeptide KFFKFFKFFK (SEQ ID NO:1) is linked to N-terminal of the PNA sequence via the ethylene glycol linker "eg1".

<400> 52

tgccatagca 10

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 October 2002 (10.10.2002)

PCT

(10) International Publication Number
WO 2002/079467 A3

(51) International Patent Classification[7]: C12N 15/10, 5/10, 15/11, C12Q 1/18, C07H 21/00, C12N 15/65

(21) International Application Number: PCT/DK2002/000208

(22) International Filing Date: 26 March 2002 (26.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2001 00523 29 March 2001 (29.03.2001) DK

(71) Applicant (*for all designated States except US*): **KØBENHAVNS UNIVERSITET** [DK/DK]; Nørregade 10, P.O. Box 2177, DK-1017 Copenhagen K (DK).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **NIELSEN, Peter, E.** [DK/DK]; Hjortevænget 509, DK-2980 Kokkedal (DK). **GOOD, Liam** [CA/SE]; Sveavägen 164, B32, S-113 46 Stockholm (SE).

(74) Agent: **PLOUGMANN & VINGTOFT A/S**; Sundkrogsgade 9, P.O. Box 831, DK-2100 Copenhagen Ø (DK).

(81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

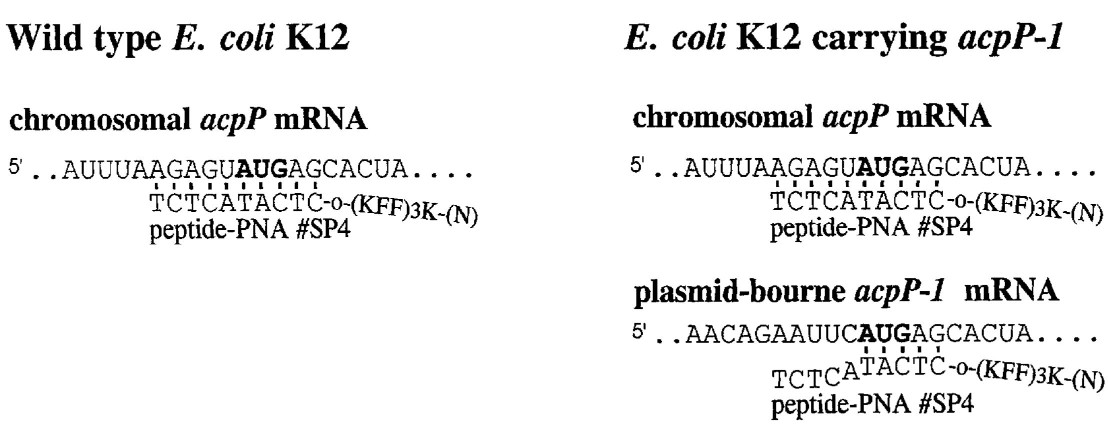
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 4 March 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** ANTIBIOTIC-FREE BACTERIAL STRAIN SELECTION WITH ANTISENSE MOLECULES



(57) **Abstract:** A new method for an antibiotic-free selection of genetically modified cells is described. It is shown that antisense molecules targeted to an essential cellular gene inhibits growth and is suited as an agent for growth selection of cells transformed with a plasmid carrying an altered version of the essential gene. The results show that antisense molecules may be used for antibiotic-free selection of desired transformed microbes when targeted against an essential microbial gene. This technology is useful in genetic engineering for research growth and isolation of transformed organisms, and for industrial growth maintenance of transformed organisms, e.g. in the production of genetically engineered proteins as an environmentally safer alternative to traditional selection methods based on antibiotics.

WO 2002/079467 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 02/00208

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12N5/10 C12N15/11 C12Q1/18 C07H21/00
C12N15/65

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 723 292 A (BELL LESLIE ET AL) 3 March 1998 (1998-03-03) column 2, line 45 - line 62 column 4, line 8 -column 5, line 46 example 1 ---	1-20,23
Y	WO 99 13893 A (NIELSEN PETER E ;GOOD LIAM (SE); ISIS PHARMACEUTICALS INC (US)) 25 March 1999 (1999-03-25) examples 2-4 ---	1-20,23
A	WO 00 44906 A (ELITRA PHARMACEUTICALS INC) 3 August 2000 (2000-08-03) example 8 page 72, line 14 -page 74, last line --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2003	10/02/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	LARSEN H J ET AL: "Antisense properties of peptide nucleic acid" BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION, vol. 1489, no. 1, 10 December 1999 (1999-12-10), pages 159-166, XP004275530 ISSN: 0167-4781 cited in the application page 161, paragraphs 2.2.,2.3. page 163, paragraph 3.3. ---	
A	MARTTI VAARA ET AL: "Group of Peptides That Act Synergistically with Hydrophobic Antibiotics against Gram-Negative Enteric Bacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 8, no. 40, 1996, pages 1801-5, XP002080197 ISSN: 0066-4804 cited in the application ---	
A	HANCOCK R E: "Peptide antibiotics" LANCET THE, vol. 349, no. 9049, 8 February 1997 (1997-02-08), pages 418-422, XP004246070 ISSN: 0140-6736 cited in the application ---	
A	DOYLE DF. ET AL.: "Inhibition of gene expression inside cells by peptide nucleic acids: effect of mRNA target sequence, mismatched bases, and PNA length." BIOCHEMISTRY 2001 JAN 9;40(1):53-64., XP002187945 ---	
A	NIELSEN PE. ET AL.: "Peptide nucleic acids as antibacterial agents via the antisense principle." EXPERT OPIN INVESTIG DRUGS 2001 FEB;10(2):331-41., XP001052987 ---	
A	WO 99 14226 A (WENGEL JESPER ;EXIQON A S (DK); NIELSEN POUL (DK)) 25 March 1999 (1999-03-25) cited in the application ---	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	GOOD LIAM ET AL: "Bactericidal antisense effects of peptide-PNA conjugates." NATURE BIOTECHNOLOGY, vol. 19, no. 4, April 2001 (2001-04), pages 360-364, XP002187946 ISSN: 1087-0156 cited in the application the whole document ---	1-29
P,X	WO 01 27261 A (WISSENBACH MARGIT ;BECK FREDERIK (DK); MALIK LEILA (DK); PANTHECO) 19 April 2001 (2001-04-19) the whole document -----	1-29

INTERNATIONAL SEARCH REPORT	International application No. PCT/DK 02/00208

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-19 and 27 (as far as in vivo method are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 30
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 30

Present claim 30 relates to a product defined by reference to a method used during its manufacture.
The claims cover all products this characteristic, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search is impossible. Consequently, no search has been carried out for claim 30.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Inf[redacted]ation on patent family members

Internat[redacted]pplication No
PCT/DK 02/00208

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5723292 A	03-03-1998	US 5871957 A	16-02-1999
		AT 78055 T	15-07-1992
		AU 600885 B2	30-08-1990
		AU 4279485 A	28-11-1985
		CA 1285505 A1	02-07-1991
		DE 3586304 D1	13-08-1992
		DE 3586304 T2	10-12-1992
		DK 235085 A	26-11-1985
		EP 0171142 A1	12-02-1986
		JP 2543497 B2	16-10-1996
		JP 61056092 A	20-03-1986
		JP 2704115 B2	26-01-1998
		JP 7067685 A	14-03-1995
		US 5527668 A	18-06-1996
		US 5700643 A	23-12-1997
		US 4931373 A	05-06-1990
WO 9913893 A	25-03-1999	US 6300318 B1	09-10-2001
		AU 9485598 A	05-04-1999
		EP 1015011 A1	05-07-2000
		JP 2001516724 T	02-10-2001
		US 6190866 B1	20-02-2001
		WO 9913893 A1	25-03-1999
WO 0044906 A	03-08-2000	AU 3584500 A	18-08-2000
		CA 2360085 A1	03-08-2000
		EP 1149166 A2	31-10-2001
		EP 1178052 A2	06-02-2002
		JP 2002535007 T	22-10-2002
		WO 0044906 A2	03-08-2000
		US 2002045592 A1	18-04-2002
WO 9914226 A	25-03-1999	AU 9063398 A	05-04-1999
		CA 2303299 A1	25-03-1999
		CN 1279687 T	10-01-2001
		WO 9914226 A2	25-03-1999
		EP 1015469 A2	05-07-2000
		JP 2002521310 T	16-07-2002
		NZ 503765 A	26-04-2002
		US 2002068708 A1	06-06-2002
WO 0127261 A	19-04-2001	AU 7773000 A	23-04-2001
		BR 0014756 A	09-07-2002
		CN 1387567 T	25-12-2002
		WO 0127261 A2	19-04-2001
		EP 1220902 A2	10-07-2002
		HU 0203465 A2	28-01-2003
		NO 20021711 A	11-06-2002